Frequency and Specificity of Antiheart Antibodies in Patients With Dilated Cardiomyopathy Detected Using SDS-PAGE and Western Blotting

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Objectives. This study was designed to investigate the organ and disease specificity of antiheart antibodies in patients with dilated cardiomyopathy.

Background. Autoimmune disease is characterized by the presence of circulating autoantibodies, and autoimmune mechanisms may play a role in the pathogenesis of dilated cardiomyopathy.

Methods. An SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) procedure followed by Western blotting was used to screen serum samples for antiheart antibodies of two immunoglobulins, IgM and IgG, from 52 patients with dilated cardiomyopathy and 48 patients with ischemic heart disease as control subjects. Use of two-dimensional gel electrophoresis followed by Western blotting and protein sequencing enabled us to identify the protein bands against which antiheart antibodies were produced in both groups of patients.

Results. Strong IgG antiheart antibodies against myocardial proteins, cross-reacting with skeletal muscle proteins, were detected in significantly more patients with dilated cardiomyopathy (n = 24 [46%]) than with ischemic heart disease (n = 8 [17%]) (p = 0.001). Patients with dilated cardiomyopathy showed a significantly greater frequency and reactivity of IgG antiheart antibodies against six myocardial proteins (molecular weight 30, 35, 40, 60, 85 and 200 kD) than did patients with ischemic heart disease. These were identified as myosin light chain 1, tropomyosin, actin, heat shock protein (HSP)-60, an unidentified protein and myosin heavy chain, respectively.

Conclusions. We detected strong IgG antiheart antibodies in significantly more patients with dilated cardiomyopathy than with ischemic heart disease. The most immunogenic band was that corresponding to HSP-60. Antibodies against HSP-60 were found in 85% and 42% of patients with dilated cardiomyopathy and ischemic heart disease, respectively, confirming our hypothesis of an immune involvement in dilated cardiomyopathy.

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In a previous limited study (7) of antiheart antibodies before and after heart transplantation, we showed a greater prevalence of weak antiheart antibodies in patients with ischemic heart disease, but the two cases of strong antiheart antibodies of the immunoglobulin IgG class were both associated with dilated cardiomyopathy. The aim of this study was to determine the organ and disease specificity of antiheart antibodies in patients with dilated cardiomyopathy or ischemic heart disease and in normal disease-free persons and then characterize the antigens involved.

Methods

Pieces of ventricular myocardium from the explanted heart of 10 recipient patients were obtained at the time of transplantation. The banding pattern from immunoblotting results was not dependent on the original diagnosis. The myocardial pieces were immediately frozen in liquid nitrogen. Skeletal muscle was obtained from patients undergoing routine cardiac surgery.

Control sera were collected from 18 laboratory workers (8 men, 10 women [mean age 32 years, range 18 to 43]).
Serum samples were collected from patients diagnosed with dilated cardiomyopathy or ischemic heart disease. All serum samples were stored at -20°C.

Patients. The dilated cardiomyopathy group included 52 patients (40 men, 12 women [mean age 42 years, range 5 to 65]). To confirm the diagnosis, all patients underwent an assessment that included a medical history, drug therapies, clinical investigations, two-dimensional echocardiography, fractional shortening, cardiac catheterization, evaluation of hemodynamic function and coronary arteriography. Left ventricular end-diastolic and end-systolic dimensions were 47 to 89 mm (mean 70.37) and 33 to 77 mm (mean 61.94), respectively. Fractional shortening was 2% to 30% (mean 12.48%), left atrial internal dimensions were 32 to 60 mm (mean 43.78) and ejection fraction was 9% to 66% (mean 31.75%).

Forty-eight patients (44 men, 4 women [mean age 55 years, range 35 to 69]) with ischemic heart disease were studied. Of these 48 patients, 18 had had a documented myocardial infarction 3 to 15 years before the time of serum sampling. The ejection fraction for this group was 10% to 45% (mean 30.8%).

Serum samples were collected from patients just before heart transplantation. There was no significant difference in hemodynamic severity between the group of patients with dilated cardiomyopathy and those with ischemic heart disease.

Eighteen normal disease-free subjects (8 men, 10 women [mean age 32, range 18 to 43]) were used as a control group for comparative purposes.

SDS-PAGE. Samples of ventricular myocardium and skeletal muscle were prepared according to the technique described by Dunn et al. (7). Ventricular myocardium and skeletal muscle proteins (total weight 25 μg) were separated on 7-cm long 10% T (total concentration of acrylamide plus bis-acrylamide in gm/100 ml) SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gels with a 3% T stacking gel.

Two-dimensional polyacrylamide gel electrophoresis. In this procedure, separations of 600-μg samples of myocardial proteins were performed using the Millipore Investigator two-dimensional electrophoresis system (8).

Western blotting. Before electroblotting, the gels were equilibrated for 30 min in transfer buffer (20 mmol/liter Tris base, 150 mmol/liter glycine). For one- and two-dimensional gels, the proteins were electrophoretically transferred to supported nitrocellulose (Hybond C Super, Amersham International) at 500 mA for 1 h or at 400 mA for 14 h, respectively.

Detection of antiheart antibodies. Nitrocellulose membranes carrying lanes of myocardial and skeletal proteins separated by SDS-PAGE and myocardial proteins separated by two-dimensional PAGE were probed according to a previously described technique (7). Serum was used at a concentration of 1/200 and secondary antibodies were used at 1/500 and 1/1,000 for the immunoglobulin classes IgG and IgM, respectively. Visualization of the protein bands to which antiheart antibodies had bound was accomplished using an enhanced chemiluminescence detection system (Amersham International). Blots were incubated with the detection reagent for 1 min and then exposed to Hyperfilm ECL for 1, 2 and 5 min. The films were developed using an automated radiograph developer.

Protein sequencing. For N-terminal protein sequencing, a two-dimensional separation of heart proteins was transferred onto a FluoroTrans membrane (Pall). The blotting buffer was 50-mmol/liter Tris base, 50 mmol/liter boric acid, pH 8.5, and the transfer was carried out at 400 mA for 14 h and then stained with Coomassie Brilliant Blue. The spots of interest were excised from the blot and placed into a New Blott cartridge (ABI) fitted to an ABI model 477A pulsed liquid protein sequencer equipped with a model 120A PTH analyzer. Proteins were sequenced using the optimized blot program (9).

Stripping membranes. For the localization of protein spots after immunoprobing, the two-dimensional blots were stripped of primary and secondary antibodies and then stained using Amido Black. The blots were incubated in stripping solution (100 mmol/liter 2-mercaptoethanol, 2% weight/volume sodium dodecyl sulfate, 62.5 mmol/liter Tris chloride, pH 6.7) for 30 min at 50°C with occasional agitation. The membranes were then washed thoroughly in phosphate-buffered saline-Tween solution and stained with Amido Black.

Isolation and purification of myofibrils. Myofibrils were isolated and prepared according to the method described by Solaro et al. (9).

Data analysis. A Fisher exact test was used to compare the positive results in both groups of patients. A p value of < 0.05 was considered statistically significant.

Results

All sera, dilated cardiomyopathy, ischemic heart disease and normal disease-free samples, whether positive or negative for antiheart antibodies, showed a background band that had a molecular weight of 70 or 50 kD, depending on whether the secondary antibody was peroxidase-conjugated IgM or IgG (Fig. 1 to 3). This band was caused by the binding of the IgM and IgG secondary antibody to their respective immunoglobulin heavy chains contained in the myocardial and skeletal muscle samples.

Normal sera showed minimal if any reactivity against myocardial and skeletal proteins. Eight samples (44%) tested negative and 10 (55%) weakly positive for IgM reactivity and 11 samples (61%) tested negative and 7 (39%) weakly positive for IgG reactivity (Fig. 1). None of the normal samples showed medium or strong antibodies of either the IgM or the IgG class. Of the 10 samples weakly positive for IgM reactivity, 4 (22%) were positive for the 35-kD band and 2 (11%) for the 200-kD band. Of the seven samples (39%)

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weakly positive for IgG reactivity, three (16%) were positive for the 35-kD band and four (22%) for the 20-kD band.

Forty-four patients (85%) with dilated cardiomyopathy and 46 patients (96%) with ischemic heart disease tested positive for antiheart antibodies of the IgM class. All 48 patients (100%) with dilated cardiomyopathy and 45 patients (96%) with ischemic heart disease tested positive for anti-heart antibodies of the IgG class. There were no significant differences between the two groups of patients in testing positive for antibodies of either the IgM or the IgG class.

The positive results were graded according to the strength of the reactions (intensity and number of reactive bands) as weak, medium and strong antiheart antibodies (Tables 1 and 2).

There were no significant differences between the two groups of patients regarding the strength of IgM antibodies (Table 1). Most patients with dilated cardiomyopathy (46%) and ischemic heart disease (50%) showed weak IgM antibodies; however, there were significant differences in the production of IgG antibodies (Table 2).

Significantly more patients with ischemic heart disease than with dilated cardiomyopathy had weak antiheart antibodies of the IgG class (Fig. 2), and significantly more patients with dilated cardiomyopathy than with ischemic heart disease had strong antiheart antibodies of the IgG class (Fig. 3). All patients with dilated cardiomyopathy tested

Table 1. Strength of IgM Antiheart Antibodies in Patients With Dilated Cardiomyopathy and Ischemic Heart Disease

<table>
<thead>
<tr>
<th>Strength of Antibody</th>
<th>Dilated Cardiomyopathy</th>
<th>Ischemic Heart Disease</th>
<th>Fisher Exact Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>−VE</td>
<td>8</td>
<td>15.5</td>
<td>2</td>
</tr>
<tr>
<td>Weak</td>
<td>24</td>
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<tr>
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<td>13</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>Strong</td>
<td>7</td>
<td>13.5</td>
<td>7</td>
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</tbody>
</table>

−VE = negative (no antiheart antibodies detected).
positive for IgG antibodies, whereas 8 patients (15.5%) with dilated cardiomyopathy tested negative for IgM antibodies.

In both patient groups, reactivity against protein bands was tabulated according to the molecular weight of the bands. Antibodies of the IgM and the IgG class were produced against 11 and 12 distinct bands, respectively. There were no differences in the frequency of IgM antiheart antibodies between the patients with dilated cardiomyopathy and ischemic heart disease (results not shown). However, the patients with dilated cardiomyopathy showed a significantly greater frequency (p < 0.05) and reactivity of IgG antiheart antibodies against six myocardial proteins (molecular weight 30, 35, 40, 60, 85 and 200 kD) (Fig. 2) than did patients with ischemic heart disease (Table 3).

Antibodies cross-reacting with skeletal muscle. All of the reactivities against the six myocardial proteins cross-reacted with skeletal muscle proteins. However, the band of reactivity against myocardial proteins was always of greater intensity than that against skeletal muscle proteins.

Four patients with dilated cardiomyopathy and seven with ischemic heart disease produced some skeletal muscle-specific bands. One of the four patients with dilated cardiomyopathy showed a skeletal muscle-specific band of the IgG class. Regarding IgG skeletal muscle-specific bands, one patient showed one band of the IgG class against a 30-kD band, one patient showed two skeletal muscle-specific bands (one of 30 kD and one of 35 kD) and one patient showed one skeletal muscle-specific band of 35 kD. Of the seven patients with ischemic heart disease showing skeletal muscle-specific bands, six showed reactivity against a 30-kD band of the IgM class and one patient showed reactivity against a 35-kD band of the IgG class.

Characterization of reactive bands. To ascertain whether any of these reactive bands were myofibrillar proteins, sera from patients with dilated cardiomyopathy were tested against a purified preparation of myofibrillar. Of all the bands listed in Table 3, the only bands of the myofibrillar protein fraction reactive with sera from patients with dilated cardiomyopathy were the 30-, 35-, 40- and 200-kD bands (Fig. 4). These bands were hence categorized as being myofibrillar proteins. On the basis of their molecular weight, likely candidates for these bands are myosin light chain 1, tropomyosin, actin, and, probably, myosin heavy chain, respectively.

Table 2. Strength of IgG Antiheart Antibodies in Patients With Dilated Cardiomyopathy and Ischemic Heart Disease

<table>
<thead>
<tr>
<th>Strength of Antibody</th>
<th>Dilated Cardiomyopathy</th>
<th>Ischemic Heart Disease</th>
<th>Fisher Exact Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>-VE</td>
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<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Weak</td>
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<tr>
<td>Medium</td>
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<td>21</td>
<td>9</td>
</tr>
<tr>
<td>Strong</td>
<td>24</td>
<td>46</td>
<td>8</td>
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</table>

*Significant. -VE = negative (no antiheart antibodies detected).

Table 3. Frequency of IgG Antiheart Antibodies in Patients With Dilated Cardiomyopathy and Ischemic Heart Disease

<table>
<thead>
<tr>
<th>Molecular Weight of Protein (kD)</th>
<th>Dilated Cardiomyopathy</th>
<th>Ischemic Heart Disease</th>
<th>Fisher Exact Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
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<tr>
<td>30</td>
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<td>44</td>
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</tr>
<tr>
<td>200</td>
<td>35</td>
<td>67</td>
<td>20</td>
</tr>
</tbody>
</table>

*Significant.
Figure 5. A two-dimensional silver-stained separation of heart proteins. The 1 represents the isoelectric focusing dimension with the acidic proteins on the left and the basic proteins on the right; 2 represents the molecular weight dimension with the high molecular weight proteins at the top and the low molecular weight proteins at the bottom of the gel.

The 30-kD band was confirmed as myosin light chain 1 (Fig. 7a), the 35-kD band as tropomyosin (Fig. 7b) and in most cases the 40-kD band as actin (Fig. 7c), but in a few cases two spots under actin were reactive with antibodies in dilated cardiomyopathy sera and these remain as yet unidentified. Antibodies against the 60-kD band reacted with two spots on the two-dimensional blots (Fig. 7d). These spots were excised from a Coomassie Blue-stained two-dimensional blot on a FluoroTrans membrane and N-terminal sequence analysis was performed. The first 20 amino acids sequenced are shown here in single letter code: AKDVKYGADARALMLQGVDL.

A search was then made for homologies with known sequenced proteins, and a 100% homology was found with heat shock protein (HSP)-60. To confirm this finding, two antibodies were obtained, one against mycobacterial HSP-65 (a gift of Dr. J. Ivanyi) and LK-1, which is against human HSP-60 (a gift of Dr. W. Van Eden). Both antibodies reacted exclusively with the two spots identified using dilated cardiomyopathy sera positive for a 60-kD band (Fig. 8). This band was hence identified as HSP-60.

The 85-kD band has also been identified on two-dimensional blots (Fig. 7e), but on sequencing it was found to have a blocked N-terminus. Further characterization of this protein will require internal protein sequence analysis.

Discussion

This study presents a systematic evaluation of antiheart antibodies in sera from patients with dilated cardiomyopathy or ischemic heart disease and normal, healthy, volunteer control subjects. Our results have demonstrated that a large number of autoantibodies are generated in patients with dilated cardiomyopathy.

The principal procedure utilized here was Western blotting. A number of studies have confirmed our own findings (7) that immunofluorescence is less sensitive than Western
Blooding in detecting antihert antibodies (6,10) and that this type of screening procedure is unlikely to identify any single cardiac antigen. Moreover, Western blotting has the advantage that reactive antigens can be subsequently identified by the use of molecular weight markers, polyclonal and monoclonal antibodies and direct protein microsequencing.

A large variety of bands were detected by Western blotting in patients with dilated cardiomyopathy. Eleven bands were reactive with IgM and 12 with IgG antibodies. These bands were also found in patients with ischémic heart disease but at significantly lower frequencies. Sera from patients with dilated cardiomyopathy were further characterized against myofibrillar proteins, likely candidates being myosin light chain 1, troponyosin, actin and myosin heavy chain. The identity of these bands was confirmed by probing two-dimensional blots of heart proteins, which revealed new, as yet unidentified, bands. The most commonly found antibodies were against a 60-kD band (Table 3); these were present in 85% and 42% of patients with dilated cardiomyopathy and ischémic heart disease, respectively. Two-dimensional gel analysis and protein sequencing revealed 100% homology of this peptide with HSP-60. Heat shock proteins are highly conserved immunogenic molecules whose cellular levels are elevated by heat, inflammatory mediators and all forms of physiologic stress (11,12). Heat shock proteins are thought to protect cells from damage during stressful conditions (13).

Antibodies and T cells reactive with heat shock proteins have been described in patients with rheumatoid arthritis, systemic lupus erythematosus and more recently in patients with schizophrenia (14) and inflammatory diseases of the bowel (15).

The presence of circulating antibodies against HSP-60 in 85% of patients with dilated cardiomyopathy is clear evidence of a damaged myocardium. The exact pattern and distribution of HSP-60 in normal and diseased myocardium has not yet been established. We are currently using both Western blotting to assess whether HSP-60 is upregulated in disease states and immunocytochemistry to investigate its precise localization on frozen specimens.

Thus, our study has positively identified reactions against the major contractile proteins as well as noncontractile proteins such as HSP-60. Although Caforio et al. (6) suggested that myosin is the major autoantigen in dilated cardiomyopathy, the present study does not support this conclusion. Antimyosin antibodies were found in 67% of patients with dilated cardiomyopathy but also in 42% of patients with ischemic heart disease. It may be that the diversity of autoantibodies found in patients with dilated cardiomyopathy is more important than reactivity of one particular specificity in the pathogenesis of dilated cardiomyopathy. We have not detected antihert antibodies reported in previous studies against the beta, adrenergic receptor (1), the adenine nucleotide translocator (3-5) or the M7 mitochondrial antigen (2). This may be explained by the use of enriched membrane and mitochondrial fractions in previous studies, in contrast to the total cardiac homogenate used in our study.

Not all antihert antibody reactivity detected was found to be cardiac specific. This is not surprising because cardiac and skeletal muscle share the majority of their proteins, varying only in the different isoforms of each protein. In humans, two types of myosin heavy chain are present in the atria and ventricles (16). These two can be classified as alpha and beta types. Immunochemical examination (16) and immunofinity chromatography (17) of human cardiac myosin has shown that myosin heavy chain-beta predominates in the ventricle and is also present in slow skeletal muscle and both are encoded by the same gene (18). It is therefore not surprising that the antibodies detected against myosin heavy chain-beta cross-react with the skeletal muscle myosin.

Actin has many isoforms (19). The sarcomeric acts, alpha-skeletal and alpha-cardiac, are expressed only in skeletal and cardiac muscle. The cardiac actin differs from skeletal muscle alpha-actin only at four amino acid residues (20), and antibodies detected by Western blotting against actin could not distinguish between the two isoforms.

Two main isoforms of tropomyosin (alpha and beta) exist in striated muscle (21,22). In the human ventricle, the alpha isoform constitutes 90.6% of the total tropomyosin (23). The fact that the cardiac alpha isoform has a primary sequence identical to that of the skeletal form (24,25) explains why one band and two bands were seen in the cardiac and skeletal lanes, respectively, and that antibodies recognized both the cardiac and the skeletal isoforms.

The major myosin alkali light chain of adult heart ventricles, myosin light chain 1, has been shown by one- and two-dimensional gel electrophoresis and partial peptide analysis to be very similar to the skeletal slow muscle isoform myosin light chain 1 (26). Venticular myosin light chain 1 has a molecular weight of approximately 28 kD, but skeletal muscle myosin light chain 1 has an approximate molecular weight of 25 kD, explaining why a corresponding band was not detected alongside the ventricular myosin light chain 1.

This cross-reactivity of antihert antibodies may explain why some of these patients have subclinical skeletal myopathy. Caforio et al. (27) studied patients with dilated cardiomyopathy and patients with hypertrophic cardiomyopathy. In ~37% of patients in both groups, there was an abnormal reduction of skeletal motor unit potential duration indicative of myogenic myopathy. It would be interesting to absorb our sera with skeletal muscle to determine which bands are specific for cardiac muscle.

A number of studies (1-6) have now documented autoantibodies against heart muscle. These observations raise three important questions: 1) Does the autoantibody profile of a patient have any diagnostic or prognostic value? 2) Are the antibodies primarily involved in development of the disease or are they formed secondary to infection or myocardial injury? 3) Are the antibodies produced as a result of the release of sequestered antigens, induction of neantigens, molecular mimicry or alteration in the host’s immune response?
responses (28,29). It would be of value to test all the putative antigens in an enzyme-linked immunosorbent assay so that quantitative data can be produced. In this way, the relative importance of the antigens as diagnostic markers of disease could be assessed. Some functional studies (30–32) have already established that antiheart antibodies can cause cardiac dysfunction. Thus, antibodies directed against the adenine nucleotide translocator enhanced intracellular calcium transport when added to isolated beating cardiac myocytes, and immunization of guinea pigs with purified adenine nucleotide translocator induced abnormal adenine nucleotide translocator–carrier function in vivo (31) and caused marked impairment in cardiac function in vivo (32).

Future work should aim at establishing whether these antibodies can be damaging against a human fetal cardiac myocyte cell line (33) or isolated adult human cardiac myocytes. We intend to elute antibodies from nitrocellulose blots to determine whether antibodies against different cardiac peptides are damaging, using isolated human cardiac myocytes as targets.

We are grateful to the British Heart Foundation for the support of this research. We thank Dr. J. Ivanyi (Hammersmith Hospital, London) and Dr. W. Van Eden (University of Utrecht, The Netherlands) for supplying the samples of the antibodies to HSP-60 heat shock protein.

References


