# 11 Clinical Implications of Anti-cardiac Immunity in Dilated Cardiomyopathy

A.L.P. Caforio, N.G. Mahon, W.J. McKenna



**Abstract.** Criteria of organ-specific autoimmunity are fulfilled in a subset of patients with myocarditis/dilated cardiomyopathy (DCM). In particular, circulating heart-reactive autoantibodies are found in such patients and symptom-free

relatives. These autoantibodies are directed against multiple antigens, some of which are expressed in the heart (organ-specific), others in heart and some skeletal muscle fibres (partially heart-specific) or in heart and skeletal muscle (muscle-specific). Distinct autoantibodies have different frequency in disease and normal controls. Different techniques detect one or more antibodies, thus they cannot be used interchangeably for screening. It is unknown whether the same patients produce more antibodies or different patient groups develop autoimmunity to distinct antigens. IgG antibodies, shown to be cardiac- and disease-specific for myocarditis/DCM, can be used as autoimmune markers for relatives at risk as well as for identifying patients in whom immunosuppression may be beneficial. Some autoantibodies may also have a functional role, but further work is needed.

## 11.1 Introduction

Autoimmune disease takes place due to the loss of tolerance to self-antigens that is maintained under physiological conditions. To be classified as autoimmune, a disease must fulfill at least two of the major criteria proposed by Witebsky and later updated by Rose (Witebsky et al. 1957; Rose and Bona 1993). There are also minor criteria, some of which are common to all autoimmune conditions. These criteria are shown in Table 1.

Circulating autoantibodies, a feature of autoimmune disease, are not always pathogenic but represent markers of ongoing tissue damage. In non-organ specific autoimmune conditions the autoantibodies are against ubiquitous autoantigens (e.g. nuclear antigens in systemic lupus erythematosus) and organ damage is generalized. In organ-specific autoimmune disease, immunopathology is restricted to one tissue or apparatus, and the antibody and/or cell-mediated immune damage is directed against autoantigens that are unique to the affected organ (e.g. thyroid peroxidase in Hashimoto's thyroiditis). An early histological finding of organ-specific autoimmunity is a mononuclear cell infiltrate in the affected target tissue, e.g. insulitis in type 1 insulin-dependent diabetes mellitus (IDDM), with inappropriate or increased expression of HLA class II and of adhesion molecules. At a later stage, inflammatory cells are reduced and fibrotic changes occur, leading to tissue atrophy and organ dysfunction (such as in Hashimoto's thyroiditis). However,

in other instances, organ-specific autoimmunity may be associated with enhanced target organ function (e.g. Basedow's disease).

Organ-specific autoimmune diseases occur as a result of genetic and environmental factors. The genetic predisposition explains why different autoimmune conditions may be associated in patients or in their relatives, as well as for the well-known feature that single autoimmune diseases often have familial aggregation. The inheritance of susceptibility is usually polygenic. Organ-specific autoimmune diseases are commonly associated with specific HLA class II antigens, but the mechanisms by which

**Table 1.** Criteria of autoimmune disease

| |
|---|
| Major |
| – Mononuclear cell infiltration and abnormal HLA expression in the target organ (organ-specific disease) or in various organs (non organ-specific disease) |
| – Circulating autoantibodies and/or autoreactive lymphocytes in patients and in unaffected family members |
| – Autoantibody and/or autoreactive lymphocytes in situ within the affected tissue |
| – Identification and isolation of autoantigen (s) involved |
| – Disease induced in animals by immunisation with relevant autoantigen, and/or passive transfer of serum, purified autoantibody and/or lymphocytes |
| – Efficacy of immunosuppressive therapy |
| Minor |
| (a) Common to all autoimmune disorders |
| – Middle-aged women most frequently affected |
| – Familial aggregation |
| – HLA association |
| – Hypogammaglobulinaemia |
| – Clinical course characterized by exacerbations and remissions |
| – Autoimmune diseases associated in the same patient or in family members |
| (b) Typical of organ-specific autoimmune disorders |
| – Autoantigens at low concentration |
| – Autoantibodies directly against organ-specific autoantigens |
| – Immunopathology mediated by type II, IV, V, VI reactions |

HLA is involved in disease predisposition are largely undefined. The majority of organ-specific autoimmune diseases are chronic and previously classified as "idiopathic". Organ- and disease-specific antibodies are detected in the affected patients. These antibodies are also found in family members years before disease development and thus identify asymptomatic relatives at risk (Bottazzo et al. 1986). Organ-specific autoimmunity is involved in a proportion of the idiopathic forms of dilated cardiomyopathy and myocarditis (MacLellan and Lusis 2003; Okazaki et al. 2003; Eriksson et al. 2003).

## 11.2 DCM and Myocarditis: Classification and Clinical Features

Dilated cardiomyopathy (DCM), is an important cause of heart failure and a common indication for heart transplantation. It is characterized by dilatation and impaired contraction of the left or both ventricles; it may be idiopathic, familial/genetic, viral, and/or immune-based (Richardson et al. 1996). Clinical presentation may be with symptoms/signs of congestive heart failure, bradyarrhythmia/tachyarrhythmia or thromboembolism. The duration of the pre-clinical phase is often uncertain. Asymptomatic DCM may be diagnosed following the detection of a systolic murmur of mitral insufficiency, of a left bundle branch block on ECG, or of enlarged cardiac chambers with systolic dysfunction on echocardiography. DCM is familial in at least 30% of cases and has adverse prognosis. The diagnosis of DCM requires the exclusion of known, specific causes of heart failure, including coronary artery disease. On endomyocardial biopsy there is myocyte loss, compensatory hypertrophy, fibrous tissue and immunohistochemical findings consistent with chronic inflammation (myocarditis) in 30%–40% of cases.

Myocarditis is an inflammatory disease of the myocardium, and is diagnosed by endomyocardial biopsy using established histological, immunological and immunohistochemical criteria; it may be idiopathic, infectious or autoimmune and may heal or lead to DCM (Aretz et al. 1985; Richardson et al. 1996; Caforio and McKenna 1996). The clinical features of myocarditis are heterogeneous. Cardiac involvement may be preceded (1–2 weeks) by a systemic flu-like illness. Myocardi-

tis may be subclinical, causing minor symptoms (palpitation, atypical chest pain), ECG abnormalities (atrioventricular conduction disturbance, bundle branch block, ST and T wave changes), or arrhythmias (paroxysmal atrial fibrillation or ventricular arrhythmias) with or without global or regional left and/or right ventricular dysfunction. Pericardial involvement commonly coexists with myocarditis. Other presentations of myocarditis include syncope, sudden death, acute right or left ventricular failure, cardiogenic shock, or DCM. A syndrome mimicking acute myocardial infarction, but with normal coronary arteries, may also occur.

Prognosis of myocarditis is thought to be good, with complete recovery in the majority of patients. However, in neonates and young children, the elderly and the debilitated, the disease is often severe, causing fulminant and fatal heart failure. Relapses may occur, and a proportion of patients will develop residual mild left ventricular dysfunction or DCM. Thus, in a patient subset, myocarditis and DCM represent the acute and chronic stages of an inflammatory disease of the myocardium, which can be viral, post-infectious immune or primarily organ-specific autoimmune (Caforio 1994; Richardson et al. 1996; Caforio and McKenna 1996).

## 11.3 Involvement of Autoimmunity in Myocarditis and DCM

Autoimmune features in human myocarditis/DCM include familial aggregation (Baig et al. 1998), a weak association with HLA-DR4 (Anderson et al. 1984), lymphocytic mononuclear cell infiltrates, abnormal expression of HLA class II and adhesion molecules on cardiac endothelium in affected patients and family members (Caforio et al. 1990b; Caforio and McKenna 1996; Mahon et al. 2002) and increased levels of circulating cytokines and cardiac autoantibodies in patients and family members (reviewed by Caforio et al. 2002). In addition, there are experimental models of both antibody-mediated and cell-mediated autoimmune myocarditis/DCM following immunization with relevant autoantigen(s) (MacLellan and Lusis 2003; Okazaki et al. 2003; Eriksson et al. 2003), the best characterized of which is cardiac myosin (Rose

2000; Kuan et al. 2000). In this chapter, we mainly focus on the clinical implications of circulating cardiac autoantibodies.

### 11.3.1 Anti-heart Autoantibodies by s-I IFL

Several researchers reported antibodies to distinct cardiac antigens in myocarditis and DCM, but the organ- and disease-specificity of these antibody types were not always evaluated (reviewed by Caforio et al. 2002). Earlier studies by standard indirect immunofluorescence (s-I IFL) described antibodies to sarcolemmal and myofibrillar antigens, but these were either cross-reactive or untested on skeletal muscle. In addition, it remained unclear whether these antibodies were disease-specific for myocarditis/DCM, because controls with other cardiac disease were not always tested. These autoantibodies were found in 12%–75% of DCM/myocarditis patients and in 4%–34% of normal control subjects (Fletcher and Wenger 1968; Camp et al. 1969; Kirsner et al. 1973; Maisch et al. 1983a,b; Table 2). The observed antibody patterns were similar to those described in rheumatic heart disease, Dressler's and post-pericardiotomy syndromes, the "diffuse" being more frequent than the "sarcolemmal-sarcoplasmic" staining pattern. A more recent study on rat heart tissue sections showed high titre (1:20) antibodies of IgG class in 59% of patients with myocarditis, 20% with DCM and in none of the normal control subjects; interestingly, these authors suggested that the three main antibody patterns ("diffuse"; "peripheral" or "sarcolemmal"; "fibrillary" or "striated") could co-exist (Neumann et al. 1990).

Using indirect s-I IFL on 4-μm-thick unfixed fresh frozen cryostat sections of blood group O normal human atrium, ventricle and skeletal muscle, and absorption with human heart and skeletal muscle and rat liver, organ-specific antibodies of IgG class were found in about one third of myocarditis/DCM patients and their symptom-free family members, 1% of patients with other cardiac disease, 3% of normal subjects and 17% of patients without cardiac disease but with autoimmune polyendocrinopathy (Caforio et al. 1990a, 1991, 1994, 1997a; Table 2). Cardiac antibodies of the cross-reactive 1 type, which exhibited partial organ-specificity for heart antigens by absorption, were also more frequently detected in DCM/myocarditis and autoimmune polyendocrinopathy than in controls. Conversely, cardiac antibodies of the cross-reactive 2 type,

**Table 2.** Anti-heart autoantibodies in myocarditis (M) and DCM

| Antibody | Antibody positive (%) Technique | M | DCM | OCD | Normals | Reference(s) |
|---|---|---|---|---|---|---|
| Muscle-specific | | | | | | |
| ASA | s-I IFL | 47* | 10 | NT | 25 | Maisch et al. 1983a,b |
| AMLA | AMC | 41* | 9 | NT | 12 | Maisch et al. 1983a,b |
| AFA | s-I IFL | 28* | 24* | NT | 6 | Maisch et al. 1983a,b |
| IFA | s-I IFL | 32* | 41* | NT | 3 | Maisch et al. 1983a,b |
| Heart-reactive | s-I IFL | 59* | 20* | NT | 0 | Neumann et al. 1990 |
| | s-I IFL | NT | 12–28 | 21–33 | 4 | Fletcher and Wenger 1968; Camp et al. 1969; Kirsner et al. 1973 |
| Anti-s.Na/K-ATPase | ELISA+Western blot | NT | 26* | NT | 2 | Baba et al. 2002 |
| Organ-specific cardiac | s-I IFL+abs | 34*,** | 26*,** | 1 | 3 | Caforio et al. 1990a, 1997a,b |
| Anti-Mitochondrial | | | | | | |
| M7 | ELISA | 13* | 31* | 10 | 0 | Klein et al. 1984 |
| ANT | SPRIA | 91*,** | 57*,** | 0 | 0 | Schultheiss and Bolte 1985; Schultheiss et al. 1990 |
| BCKD-E2 | ELISA | 100*,** | 60*,** | 4 | 0 | Ansari et al. 1994 |
| Anti-laminin | ELISA | 73 | 78 | 25–35 | 6 | Wolff et al. 1989 |

AFA, anti-fibrillary antibody; AMC, antibody-mediated cytotoxicity; AMLA, anti-myolemmal antibody; ASA, anti-sarcolemmal antibody; IFA, anti-interfibrillary; LBI, ligand-binding inhibition; NT, not tested; OCD, other cardiac disease (other abbreviations as in text) $^{*}p < 0.05$ vs normals $^{**}p < 0.05$ vs OCD +abs, +absorption

**Table 2.** (continued)

| Antibody | Antibody positive (%) Technique | M | DCM | OCD | Normals | Reference(s) |
|---|---|---|---|---|---|---|
| Anti-$\beta_1$ Receptor | | | | | | |
| Inhibiting | LBI | NT | 30–75*,** | 37 | 18 | Limas et al. 1989, 1991 |
| | ELISA | NT | 31*,** | 0 | 12 | Magnusson et al. 1990, 1994 |
| Stimulating | Bioassay | 96*,** | 95*,** | 8 | 0 | Wallukat et al. 1991 |
| | ELISA | NT | 38** | 6 | 19 | Chiale et al. 1995 |
| | ELISA | NT | 26*,** | 10 | 1 | Jahns et al. 1999 |
| Anti-M2 Receptor | ELISA | NT | 39* | NT | 7.5 | Fu et al. 1993 |
| Anti-α-and -β-MHC | Western blot | NT | 46*,** | 8 | 0 | Caforio et al. 1992 |
| Anti-MLC 1v | Western blot | NT | 35 | 25 | 15 | Caforio et al. 1992 |
| Non myofibrillar | Western blot | NT | 46*,** | 17 | 0 | Caforio et al. 1992 |
| Anti-MHC | Western blot | NT | 67** | 42 | NT | Latif et al. 1993 |
| Anti-MLC 1 | Western blot | NT | 17** | 0 | NT | Latif et al. 1993 |
| Anti-tropomyosin | Western blot | NT | 55** | 21 | NT | Latif et al. 1993 |
| Anti-actin | Western blot | NT | 71** | 21 | NT | Latif et al. 1993 |
| Anti-HSP-60 | Western blot | NT | 85** | 42 | NT | Latif et al. 1993 |
| Anti-HSP-60, 70 | Western blot | NT | 10–14** | 1–2 | 3 | Portig et al. 1997 |
| Anti-β-MHC | ELISA | 37*,** | 44*,** | 16 | 2.5 | Lauer et al. 1994 |
| Anti-α-MHC | ELISA | 17*,** | 20*,** | 4 | 2 | Goldman et al. 1995; Caforio et al. 1997a,b |

AFA, anti-fibrillary antibody; M, myocarditis; AMC, antibody-mediated cytotoxicity; AMLA, anti-myolemmal antibody; ASA, anti-sarcolemmal antibody; IFA, anti-interfibrillary; LBI, ligand-binding inhibition; NT, not tested; OCD, other cardiac disease (other abbreviations as in text) * $p < 0.05$ vs normals ** $p < 0.05$ vs OCD +abs, +absorption

which were entirely skeletal muscle cross-reactive by absorption, were found in similar proportions among groups. No patients with Dressler's or post-pericardiotomy syndromes, or active rheumatic heart disease, were included in these studies (Caforio et al. 1990a, 1991, 1997a). The s-I IFL patterns (figures in Caforio et al. 1990a ; Betterle et al. 1997) were as follows:

1. "*Organ-specific*". Sera were observed which gave diffuse cytoplasmic staining of both atrial and ventricular myocytes. The staining was stronger in atrial than in ventricular myocytes. These sera were negative on skeletal muscle. The titre range of these antibodies was 1/10 to 1/80 on atrial tissue and 1/10 to 1/20 on ventricular tissue. All positive sera contained antibodies of IgG class and 10% of IgM class. Organ-specific cardiac antibodies titres fell after absorption with heart homogenate, but were not affected by incubation with skeletal muscle or rat liver.
2. "*Cross-reactive 1*" or "*Partially Organ-specific*". Antibodies which gave a fine striational staining pattern on atrium and to a lesser extent on ventricle, but were negative or only weakly stained skeletal muscle sections, were classified as "cross-reactive 1" or "partially organ-specific". Their titres ranged from 1/20 to 1/80 on the atrial substrate and from 1/10 to 1/40 on ventricular tissue. All positive sera contained antibodies of IgG class and few also contained IgM antibodies. Antibody titres in the sera classified as "cross-reactive 1" or "partially organ-specific" were reduced to the same extent after absorption with heart and with skeletal muscle, and were not affected by absorption with rat liver.
3. "*Cross-reactive 2*". Antibodies which gave a broad striational pattern on longitudinal sections of heart and skeletal muscle were classified as "cross-reactive 2". This pattern had been previously found in 30%–40% of sera from myasthenia gravis patients without thymoma and in all myasthenic patients with thymoma (Zweiman and Arnason 1987). These striational antibodies have been shown to react with the A band of the myofibrils of striated muscle, and to cross-react with thymus myoepithelial cells. Cardiac antibodies in the sera classified as "cross-reactive 2" were absorbed by human skeletal muscle and to a lesser extent by heart tissue, but not by rat liver.

### 11.3.2 Anti-heart Autoantibodies by s-I IFL: Technical Aspects and Suggested Nomenclature

1. O blood group human heart and skeletal muscle are employed to avoid false-positive reactions due to heterophile or anti-ABO antibodies. Testing sera on skeletal muscle is necessary to differentiate heart-specific (organ-specific) from cross-reactive patterns (positive on heart and skeletal muscle), and on rat liver and kidney to detect nonorgan-specific mitochondrial or smooth muscle antibodies which give false positive "muscle reactive" IFL (Nicholson et al. 1977; Caforio et al. 1990a; Betterle et al. 1997). The pattern defined as "intermyofibrillary" is rare and might represent anti-mitochondrial antibodies (Nicholson et al. 1977). A pseudo-sarcolemmal or "endomysial" interstitial pattern can be observed on some tissue substrates (heart and muscle). It lacks species and tissue specificity, gives a "brush border" staining on proximal tubules of rat kidney and it represents a false-positive reaction (Nicholson et al. 1977; Betterle et al. 1997).
2. Several studies suggest that there is not a pure "sarcolemmal" or "peripheral" pattern; in fact, sera giving "striated" patterns seem to react more intensely with the periphery of the myofibre if the section does not include longitudinally cut fibres (Nicholson et al. 1977; Neumann et al. 1990; Caforio et al. 1990a; Betterle et al. 1997). It is important that the section includes longitudinally cut fibres, in order to identify "striated" patterns not visible on transverse sections.
3. It is important to use standard positive and negative control sera titrated up to end-dilution in every assay, to minimize inter-assay variability (Caforio et al. 1990a; Betterle et al. 1997).
4. New potentially organ-specific patterns should be confirmed as heart-specific by absorption (Caforio et al. 1990a; Betterle et al. 1997). If absorption is not performed, patterns of anti-heart autoantibodies by s-I IFL should be classified according to those already described and characterized (Nicholson 1977; Caforio et al. 1990a; Betterle et al. 1997), as follows:

   (a) "*Diffuse*" (also defined as "diffuse- sarcoplasmic" or "organ-specific")

(b) "*Striated, non-myasthenic*" (also defined as "cross-reactive type 1", or "partially organ-specific" or "anti-fibrillary")
(c) "*Striated, myasthenic*" (also defined as "cross-reactive type 2")
(d) "*Diffuse/striated, non-myasthenic*", if a "striated, non-myasthenic" pattern is superimposed on a diffuse sarcoplasmic stain, resulting in a combination of patterns a and b
(e) "*Anti-intercalated disks*", isolated or in combination with diffuse or striated stain

### 11.3.3 Autoantibodies to Myosin Heavy Chain and Other Autoantigens by Immunoblotting Techniques

Two of the autoantigens recognized by the cardiac autoantibodies detected by IFL were identified as α- and β-myosin heavy chain (MHC) isoforms, as well as ventricular light chain type 1 (MLC-1v), by Western blotting; several bands due to unknown antigens were also detected in DCM-positive sera (Caforio et al. 1992). These unknown antigens had an apparent molecular weight of 30–35 kDa in 50% of positive sera, 55–60 kDa in 21%, 100 kDa in 14% and 130–150 kDa in 14% (Caforio et al. 1992). The β-MHC is expressed in slow skeletal and ventricular myosin and is therefore only partially cardiac specific. The α-isoform is expressed solely within the atrial myocardium. Antibodies to this molecule represent organ-specific cardiac autoantibodies. The identification of α- and β-MHC as autoantigens in human DCM parallels what is seen in the experimental model of autoimmune myocarditis/DCM (Neu et al. 1987; Smith and Allen 1991) and in human myocarditis (Caforio et al. 1997a,b; Neumann et al. 1990; Lauer et al. 2000). The finding of anti-MHC and MLC-1v antibodies of IgG class in DCM patients has been confirmed by several groups using Western blotting (Latif et al. 1993) or enzyme-linked immunosorbent assay (ELISA) (Limas et al. 1995; Goldman et al. 1995; Michels et al. 1994). A recent study has suggested that the disease-specific anti-myosin antibodies in DCM sera are mainly of IgG3 subclass (Warraich et al. 1999). By Western blot, antibodies to heat shock protein-60 (HSP-60), tropomyosin and actin have also been found more frequently in DCM than in ischaemic heart disease controls, but normal sera were not tested (Latif et al. 1993; Table 2). Others (Portig et al. 1997) found antibodies to HSP-60 and -70 at a higher

frequency in DCM sera than in ischaemic or normal control subjects (Table 2). Latif et al. developed a microcytotoxicity assay and showed complement-mediated cytotoxic activity of DCM sera containing anti-heart antibodies by Western blot (Latif et al. 1994). DCM, ischaemic and normal control sera were screened used W1, a transformed human fetal cardiac cell line, and also EA.hy 926, an endothelial cell line, and IRB3, a fibroblast cell line. In the presence of complement, sera from 28 (62%) DCM patients showed greater killing of the W1 cell line as compared to sera from 13 (30%) of ischaemic patients ($p < 0.005$) and 3 (15%) normal subjects. Only 1 DCM patient showed killing of EA.hy 926 cell line, and 1 ischaemic showed killing of the fibroblast cell line. These in vitro data suggest that a complement-dependent, antibody-mediated mechanism of damage to cardiac myocytes may contribute to the pathogenesis of DCM.

### 11.3.4 Autoantibodies to Sarcolemmal Na-K-ATPase

A recent study, using porcine cerebral cortex Na-K-ATPase as an antigen in ELISA and as a substrate in enzyme activity measurement, tested sera from 100 DCM patients and 100 healthy individuals and found anti-Na-K-ATPase autoantibodies in 26% of DCM and in 2% of normal subjects (Baba et al. 2002; Table 2). Western blots showed that the antibodies recognized the α-subunit, and 3H-ouabain bindings in the presence of patient IgG showed that the dissociation constant was higher in DCM patients with antibodies than in those without, suggesting biological activity for the antibody. By multiple regression analysis, the presence of anti-Na-K-ATPase autoantibodies was an independent predictor for the occurrence of ventricular tachycardia. Cardiac sudden death was independently predicted by the presence of antibodies, as well as poor systolic function. The authors speculated that these antibodies may lead to electrical instability, because of abnormal $Ca^{2+}$ handling by reduced Na-K-ATPase activity, and delayed afterdepolarizations via reverse-mode operation of the $Na^{+}/Ca^{2+}$ exchanger, resulting from increased intracellular $Na^{+}$ concentrations. Although this represents an interesting hypothesis, no definitive conclusions on the functional role of these antibodies can be drawn at present. It remains to be seen whether these antibodies are disease-specific for DCM, since no controls with

heart failure from other aetiologies were studied. It is worth noting that sarcolemmal Na-K-ATPase does not seem to fulfill the strict criteria of organ-specific cardiac autoantigens: the α-1 subunit isoform is expressed in most tissues, the α-2 is predominant in skeletal muscle and can be detected in brain and heart, the α-3 is found in excitable tissues and the α-4 in testis (Urayama et al. 1989; Muller-Ehmsen et al. 2001). Similarly, the β-1 subunit isoform is fairly ubiquitous, whereas the β-2 and β-3 subunit isoforms are mostly found in skeletal muscle, neural tissues, lung and liver. In human heart, only α-1β-1, α-2β-1 and α-3β-1 heterodimers are present, and are thought to be involved in the actions of cardiac glycosides (Schwinger et al. 1999).

### 11.3.5 Autoantibodies to Mitochondrial and to Extracellular Matrix Antigens

Using ELISA, autoantibodies against laminin, a large basement membrane glycoprotein, were found in 73%–78% of myocarditis/DCM patients and in 6% of normal subjects; the authors did not include ischaemic heart disease controls, but they reported unpublished data indicating 25%–35% prevalence in coronary artery disease (Wolff et al. 1989; Table 2). Antibodies against distinct mitochondrial antigens – the M7 (Klein et al. 1984; Otto et al. 1998), the adenine nucleotide translocator (ANT) (Schultheiss and Bolte 1985; Schultheiss et al. 1990) and the branched chain α-ketoacid dehydrogenase dihydrolipoyl transacylase (BCKD-E2) (Ansari et al. 1994) and other respiratory chain enzymes (Pohlner et al. 1997) have also been detected. The M7 antibodies, detected by ELISA, were of IgG class and were found in 31% of DCM patients, 13% of those with myocarditis, 33% of controls with hypertrophic cardiomyopathy, but not in control subjects with other cardiac disease, other immune-mediated disorders or in normal subjects (Klein et al. 1984; Table 2). The test antigen was represented by different sub-cellular and sub-mitochondrial beef heart preparations; sera were also tested on sub-mitochondrial particles from pig kidney and rat liver. Using a indirect micro solid-phase radio immunoassay (SPRIA) and ANT, a protein of the internal mitochondrial membrane, purified from beef heart, liver and kidney as antigen, anti-ANT antibodies were found in 57%–91% of myocarditis/DCM sera, and in no controls with

ischaemic heart disease or in normal subjects (Schultheiss et al. 1985, 1990; Table 2). Mitochondrial antigens have generally been classified as nonorgan-specific (Bottazzo et al. 1986; Rose et al. 1993). However, the heart-specificity of the M7 antibodies was shown by absorption studies, whereas these were not performed with the ANT and the BCKD-E2 antibodies. Experimentally induced affinity-purified anti-ANT antibodies cross-reacted with calcium channel complex proteins of rat cardiac myocytes, induced enhancement of transmembrane calcium current and produced calcium-dependent cell lysis in the absence of complement (Schultheiss et al. 1988, 1990). The authors suggested that such an enhancing effect of the antibodies in patients might lead to impaired function of the ANT, imbalance of energy delivery and demand within the myocyte, and subsequent cell death in vivo. The presence of this mechanism of antibody-dependent cell lysis has not been shown using the antibodies present in patients' sera.

### 11.3.6 Autoantibodies to β-Adrenergic and M2-Muscarinic Receptors

Several groups have demonstrated antibodies against the $\beta_1$-adrenoceptor (Wallukat et al. 1991; Limas et al. 1989; Limas and Limas 1991; Magnusson et al. 1990, 1994). Using a binding inhibition assay (inhibition of marked [$^3$H]dihydroalprenolol binding to rat cardiac membranes), a significant inhibitory activity, attributed to anti-$\beta_1$-adrenoceptor antibodies of IgG class, was found in 30%–75% of DCM sera, 37% of ischaemic or valvular heart disease controls and 18% of sera from normal subjects (Limas et al. 1989; Limas and Limas 1991). Positive DCM sera were also found to immunoprecipitate β-adrenoceptors from solubilized cardiac membranes. Antibody positive sera induced sequestration and endocytosis of $\beta_1$-receptors predominantly dependent on the β-receptor kinase, and selectively inhibited isoproterenol-sensitive adenylate cyclase activity (Limas et al. 1989; Limas and Limas 1991). Magnusson et al. (1990), using as antigens synthetic peptides analogous to the sequences of the second extra cellular loop of $\beta_1$- and $\beta_2$-adrenergic receptors by ELISA, found antibodies in 31% of DCM patients, 12% of normal subjects and in none of the controls with other cardiac disease. The antibodies from

DCM sera exhibited inhibitory activity of isoproterenol binding to the β-adrenergic receptor.

Other studies showed that, when analysed in a functional test system of spontaneously beating neonatal rat myocytes, antibody-positive DCM sera (Wallukat et al. 1991; Jahns et al. 1999, 2000) or the affinity-purified $\beta_1$-receptor antibodies (Magnusson et al. 1994) increased the beating frequency of isolated myocytes in vitro. $\beta_1$-Blocking drugs (propranolol, bisoprolol and metoprolol) inhibited the effect of the antibodies. These workers reported that the stimulating anti-$\beta_1$-receptor antibodies were present in 96% of myocarditis and 26%–95% of DCM sera, in 8%–10% of controls with ischaemic heart disease and 0%–19% of normal subjects (Table 2). They also suggested that this antibody-mediated stimulation of the $\beta_1$-receptor, observed in vitro, could occur in vivo and account for the accelerated decline in ventricular systolic function in some myocarditis/DCM patients.

Fu et al. (1993), using as antigen a synthetic peptide analogous to the 169–193 sequence of the second extra cellular loop of human M2 muscarinic receptors and an ELISA method, showed anti-M2 antibodies in 39% of DCM sera and 7% of the normal subjects (Table 2). The presence of the anti-M2 antibodies correlated with that of anti-β-receptor antibodies. A limitation of work involving the anti-receptor antibodies is that few disease controls have been studied. These receptors are not organ-specific cardiac autoantigens; in fact, their distribution is not restricted to the heart, and there are no cardiac-specific isoforms (Elalouf et al. 1993; Eglen et al. 1994).

## 11.4 Cardiac-Specific Antibodies in Myocarditis/DCM: Clinical Implications and Potential Functional Role

The presence of organ- and disease-specific cardiac antibodies of IgG class against myosin and other antigens supports the involvement of autoimmunity in at least one third of myocarditis/DCM patients (Caforio et al. 1990a; Neumann et al. 1990; Latif et al. 1993; Michels et al. 1994). These antibodies were associated with shorter duration and minor severity of symptoms, as well as with greater exercise capacity at diagnosis (Caforio et al. 1990a, 1997b, 2001). In many patients who

were antibody positive at diagnosis, these markers became undetectable at follow-up (Caforio et al. 1997b). These findings suggest that cardiac-specific autoantibodies are early markers. The absence of antibodies at diagnosis in some patients could indicate that cell-mediated mechanisms are predominant and/or that autoimmunity is not involved; since the pre-clinical stage in DCM may be prolonged, it might also relate to reduction of antibody levels with disease progression (Caforio et al. 1997b). These findings have been obtained using standard autoimmune serology techniques, in particular s-I IFL, ELISA and immunoblotting, and have been confirmed by several groups (Neumann et al. 1990; Latif et al. 1993; Michels et al. 1994; Limas et al. 1995). The low frequency of cardiac-specific antibodies in control patients with heart dysfunction not due to myocarditis/DCM (Caforio 1990; Caforio et al. 1994; Goldman et al. 1995) and the decrease in antibody titres in advanced DCM (Caforio et al. 1997b, 2001) suggest that these markers are not epiphenomena associated with tissue necrosis of various causes, but represent specific markers of immune pathogenesis. The role of inflammatory cytokines (e.g. the IL-2/sIL-2R system) as markers of T lymphocyte activation in immune-mediated myocarditis/DCM and its relation to cardiac autoantibodies is a controversial issue (Limas et al. 1995; Caforio et al. 2001). Limas (1995) found that high-titre anti-$\beta_1$-receptor antibodies were more common among DCM patients with abnormal sIL-2R serum levels. Others found no association between the autoantibodies found by IFL and the anti-α-myosin antibodies detected by ELISA and sIL-2R levels (Caforio et al. 2001). sIL-2R may be related with distinct autoantibody specificities, e.g. in Graves' disease high sIL-2R was associated with anti-TSH receptor autoantibodies, but was unrelated to the autoantibodies to intracellular antigens (anti-microsomal and anti-thyroglobulin; Balazs and Farid 1991). The same may apply to DCM, high sIL-2R being present in association with antibodies to extracellular, e.g. the anti-$\beta_1$-receptor, rather than intracellular antigens, e.g. α-myosin and the other antigens involved in the IFL reaction. The cardiac-specific autoantibodies found by IFL and the anti-α-myosin antibodies detected by ELISA were found in similar proportions of patients with DCM and with biopsy-proven myocarditis according to the Dallas criteria, included in the Myocarditis Treatment Trial (Mason et al. 1995), suggesting that conventional histology does not distinguish

between patients with and without an ongoing immune-mediated process in myocarditis/DCM (Caforio et al. 1997a). The Myocarditis Treatment Trial failed to show an improvement in survival in biopsy-proven myocarditis with immunosuppressive therapy; however, no immunohistochemical or serological markers (e.g. increased HLA expression on myocardial biopsy and/or detection of cardiac-specific autoantibodies in the serum in the absence of viral genome in myocardial tissue) were used to identify those patients with immune-mediated pathogenesis in whom immunosuppression could have been beneficial (Mason et al. 1995). Interestingly, a recent randomized, placebo-controlled study in DCM patients with HLA up-regulation on endomyocardial biopsy showed long-term benefit with immunosuppressive treatment (Wojnicz et al. 2001). Myocarditis/DCM patients with cardiac-specific autoantibodies should also be included in future trials of immunosuppressive therapy.

Myosin fulfilled the expected criteria for organ-specific autoimmunity, in that immunization with cardiac but not skeletal myosin reproduced, in susceptible mouse strains, the human disease phenotype of DCM (Neu et al. 1987; Smith et al. 1991). In this respect, less experimental data are available with other autoantigens. However, autoimmune diseases are often polyclonal, with production of autoantibodies to different autoantigens. Some of these autoantigens are involved earlier in disease and are more closely related to primary pathogenetic events compared to those which play a role in secondary immunopathogenesis (Rose et al. 1993). Both experimental and clinical evidence, in particular the multiplicity of autoantibody specificities identified so far (Table 2), exists that this also applies to myocarditis/DCM. Myosin is an intracellular protein, thus there are two major hypotheses – which may be not mutually exclusive – to explain interruption of tolerance to this autoantigen. These include molecular mimicry, since cross-reactive epitopes between cardiac myosin and infectious agents have been found, and myocyte necrosis due to viral infection or other tissue insults (Horwitz et al. 2000; Galvin et al. 2000; Rose 2000). Both mechanisms would explain the association of viral infection with autoimmune myocarditis/DCM. Infection with coxsackie B3 (CB3) virus triggers antimyosin reactivity and autoimmune myocarditis in many mouse strains, and immunization with cardiac myosin induces disease in the same susceptible

strains (Neu et al. 1987; Smith et al. 1991). In some strains, such as Balb/c mice, CB3 virus-induced or myosin-induced myocarditis is T cell-mediated (Smith et al. 1991), whereas in other strains, such as DBA/2 mice, it is an antibody-mediated disease (Kuan et al. 2000). The same may apply to humans, so that the antimyosin antibodies may be directly pathogenic in some (Lauer et al. 2000) but not all patients with myocarditis/DCM (Caforio et al. 1997b) according to different immunogenetic backgrounds, isotype (Kuan et al. 2000) and/or subclass specificity of these antibodies (Warraich et al. 1999).

In relation to the proposed functional role of antibodies not against myosin, e.g. the anti-b and M2 receptor and some of anti-mitochondrial antibodies (Table 2) in man, passive transfer of the myocarditis/DCM phenotype to genetically susceptible animals by antibody-positive patients' sera would provide conclusive evidence for antibody-mediated pathogenesis. Non-antigen-specific IgG adsorption has recently been used in DCM patients with high titre antibodies to the $\beta_1$-receptor, and it has been suggested that it has beneficial clinical effects, accompanied by undetectable antibody titres during follow-up (Muller et al. 2000). This does not indicate a direct pathogenic effect of the anti-$\beta_1$-receptor antibodies. The adsorption technique used was non-antigen specific; in addition, in antibody-mediated disorders the antibody titres rise again at the end of plasmapheresis. However, the authors have recently provided new evidence in favour of the possibility that the beneficial effect of immunoadsorption is related to removal of pathogenic cardio-depressant autoantibodies of IgG3 subclass, although no conclusion is possible on the potential pathogenic role of a specific autoantibody (e.g. $\beta_1$-receptor antibody); Schimke et al. 2001; Felix et al. 2002; Staudt et al. 2002). It may be that this technique has a favourable immunomodulatory/immunosuppressive effect; in addition, IgG substitution performed after immunoadsorption to avoid infective complications of unselective IgG depletion, may have contributed to the observed haemodynamic improvement (Mann 2001; Gullestad et al. 2001). Thus, randomized studies are warranted. This does not undermine the possible role of any of the described antibodies (Table 2) as predictive markers. Subjects classified as negative for one antibody may be positive for another and combined testing may be advantageous. To this end, standardization of nomenclature and protocols for antibody detection and exchange of sera

among laboratories currently assessing the individual antibodies will be useful.

In conclusion, several groups have shown that a subset of patients with myocarditis/idiopathic DCM and of their symptom-free relatives has circulating heart-reactive autoantibodies. These autoantibodies are directed against multiple antigens, some of which are strictly expressed in the myocardium (e.g. organ-specific for the heart), others are expressed in heart and skeletal muscle (e.g. muscle-specific). Distinct autoantibodies also have different prevalence in disease and normal controls (e.g. by IFL the organ-specific antibodies are disease-specific for DCM, some of the muscle-specific antibodies are not). Different antibody techniques detect one (e.g. ELISA for myosin or for anti-receptor antibodies) or more antibody specificities (e.g. indirect IFL), thus they cannot be used interchangeably as screening tools. Antibody frequency in DCM vs controls is expected to be different using distinct techniques; at present it is unknown whether the same subset (30%–40%) of patients produce more than one antibody or different patient groups develop autoimmunity to different antigens. Antibodies of IgG class, which are shown to be cardiac and disease-specific for myocarditis/DCM, can be used as reliable markers of autoimmune pathogenesis for identifying (1) patients in whom immunosuppression and/or immunomodulation therapy may be beneficial and (2) their relatives at risk. Some of these autoantibodies may also have a functional role in patients, as suggested by in vitro data as well as by preliminary clinical observations, but further work is needed to clarify this important issue.

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