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The Shift in the Myocardial Adenine Nucleotide Translocator Isoform Expression Pattern Is Associated With an Enteroviral Infection in the Absence of an Active T-Cell Dependent Immune Response in Human Inflammatory Heart Disease

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OBJECTIVES This study evaluates the relevance of an enteroviral infection and the intramyocardial T-cell immune response for the alteration in the adenine nucleotide translocator isoform transcription pattern (ANTitp) in patients suspected of having myocardial inflammation.

BACKGROUND The ANT, the only mitochondrial carrier for ADP and ATP, plays a significant role in the energy metabolism and is involved in the apoptosis process. Its function and expression were found to be altered in the myocardium of patients with dilated cardiomyopathy and myocarditis.

METHODS The ANTitp was analyzed in endomyocardial biopsies from 53 patients with clinically suspected inflammatory heart disease (csIHD). Enteroviral RNA was detected in the biopsies using the reverse transcripted polymerase chain reaction technique. The activation of the cellular immune system was assessed by the quantification of T-lymphocytes employing immunohistochemistry.

RESULTS The ANTitp was found to be altered in 21 csIHD patients. Enteroviral genome was found in the heart of 71.4% of these patients, but only 37.5% of the patients with a normal ANTitp were virus-positive ($p < 0.02$). The infiltration with $CD3^+$, $CD45R0^+$ and $CD8^+$ T-cells was substantially lower in myocardial specimens with an altered ANTitp than in biopsies with a normal ANTitp. Combining the data, an altered ANTitp was primarily found in virus-positive heart tissue, which was less infiltrated with lymphocytes or not at all.

CONCLUSIONS An enteroviral infection is linked to changes in the ANT isoform expression in human heart tissue, which shows little or no evidence of an active T-cell dependent immune response. These results make a contribution to a better understanding of the pathophysiology of enterovirus-induced human inflammatory heart disease. (J Am Coll Cardiol 2000;35: 1778–84) © 2000 by the American College of Cardiology

Enterovirus, especially Coxsackie B (CVB) subtypes, are well known viral pathogens found in inflamed human hearts (1,2). Coxsackie virus is a member of the picornavirus family. In adults, CVB infections can cause active myocarditis with acute heart failure, which in most cases heal up spontaneously within the first four months. However, in some cases chronic heart failure may supervene, leading to the clinical diagnosis of dilated cardiomyopathy (DCM).

Many studies analyzed the interaction between the viral infection and the immune system and its relevance for the development of the heart failure (3,4). However, less is known about the interaction of the viral infection and intracellular biochemical and molecularbiological processes in the infected heart tissue.

We recently reported an alteration in the function and expression of the adenine nucleotide translocator (ANT) in the myocardium of patients suffering from myocarditis or DCM (5). The ANT is the only transport system that enables the transfer of the energy-rich phosphates ADP and ATP across the inner mitochondrial membrane (6). Therefore, it represents the key link between the ATP production in the mitochondria and the ATP consumption in the cytosol. In addition, the ANT is significantly involved in the apoptosis process, which is activated in inflamed hearts (7). The ANT is a homodimeric protein and is encoded by three distinct genes, designated as ANT1, ANT2 and ANT3

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Abbreviations and Acronyms

ANT = adenine nucleotide translocator
ANTitp = adenine nucleotide translocator isoform transcription pattern
CBV = Coxsackie B virus
csIHD = clinically suspected inflammatory heart disease
DCM = dilated cardiomyopathy
EF = ejection fraction
HPF = high power field
ICM = ischemic cardiomyopathy

(8,9). These genes were shown to be differently expressed in various human tissues (10,11). The ANT function was found to be impaired, and the total ANT-protein was upregulated in heart tissue of patients suffering from DCM (12). This dysfunction was accompanied by an alteration in the expression profile of the three ANT isoforms (13). The ANT isoform shift was characterized by an increased ANT1 and a decreased ANT2 proportion. These changes were exclusively found in myocardial specimens of patients with DCM and inflammatory heart disease but not with ischemic or valvular/hypertrophic heart disease. Consequently, changes in the function and expression of the ANT are not a general phenomena of heart failure but seemed to be specifically linked to viral induced heart disease.

A reduced ANT function was also observed in Coxsackie B3 infected A/J mice, which was closely correlated to an impaired heart function, indicating a potential pathophysiological significance of the ANT in viral heart disease (14). This study analyzed the relevance of the enterovirus and the T-cell dependent immune response for the alteration in the ANT isoform transcription pattern in human inflammatory heart disease.

METHODS

Study groups. Fifty-three patients clinically suspected of having inflammatory heart disease (csIHD) were enrolled in this study (Table 1). Myocardial inflammation was presumed due to a typical history of previous viral infection at the time of the onset of cardiac disease. The duration of symptoms amounted to an average of 11 ± 14 months. Cardiac symptoms, such as cardiac arrhythmia, electrocardiographic changes and reduced exercise were observed. All patients had left ventricular dysfunction with an ejection fraction (EF) <50% or a regional dysfunction with EF >50%. The patients were examined by noninvasive and invasive techniques including echocardiography, left ventriculography and right heart catheterization. Coronary, hypertensive and valvular heart disease as well as restrictive and constrictive heart disease were excluded as a cause for the left ventricular dysfunction.

Endomyocardial right ventricular biopsies were taken from the right ventricular septum of each patient by standard percutaneous transvenous right femoral approach using a Cordis bioptome (Cordis, Haan, Germany).

Table 1. Clinical, Histological, Immunohistological and Hemodynamic Characteristics of Patients With Clinically Suspected Inflammatory Heart Disease (n = 53)

Age (yrs)	47 ± 14 (20–73)
Gender	
Women	18
Men	35
Duration of symptoms (months)	11.4 ± 14 (1–41)
Left ventriculographic variables	
Ejection fraction (%)	48 ± 21
Cardiac index (l/min/m^2)	2.9 ± 1
End-diastolic volume index (ml/m^2 BSA)	143 ± 68
Stroke volume index (ml/m^2 BSA)	60 ± 22
Left ventricular end-diastolic pressure (mm Hg)	15 ± 8
Echocardiographic parameters	
Left ventricular end-diastolic diameter (mm)	63 ± 12 (40–85)
Presence of enteroviral RNA	27
Histology	
Acute myocarditis	2
Borderline myocarditis	9
Immunohistology	
CD3$^+$ cells >7 per mm^2 (20)	25

Data are shown as mean ± SD or as number of patients. BSA = body surface area.

It had previously been shown that the myocardial ANT isoform pattern of patients suffering from ischemic cardiomyopathy (ICM) did not differ from that of healthy people (13). Therefore, explanted heart tissue from 22 patients with ICM were used as controls. Samples were taken directly after removal of the heart and immediately frozen in liquid nitrogen. The specimens were stored at −80°C awaiting analysis.

All procedures were performed in accordance with ethical standards and with the Helsinki Declaration of 1975. All patients gave their informed consent for all of the invasive studies performed.

Determination of ANT isoform-specific mRNA proportions. The ANT isoform transcription pattern was determined according to the method previously described in detail by Dörner et al. (10).

Histology. Hematoxylin-eosin staining of paraffin sections was carried out and analyzed according to standard methods (15).

Immunohistological analysis. Cryostat sections were analyzed for infiltrating T-lymphocytes, as previously published (16). In brief, biopsy specimens were covered with TissueTec embedding medium (Slee, Mainz, Germany) and snap-frozen in methylbutane precooled in liquid nitrogen; 5 μm thick frozen sections were placed onto 10% poly-L-lysine precoated slides. After blocking with 20%

fetal calf serum, tissue sections were incubated with monoclonal antibodies directed against $CD3^+$ (T-cells), $CD4^+$ (helper/inducer T-cells), $CD8^+$ (suppressor/cytotoxic T-cells) and $CD45R0^+$ (activated T-lymphocytes and memory T-cells) (Dianova, Germany). Detection of bound antibodies was performed using peroxidase-conjugated antimouse and 3-amino-9-ethylcarbazol. Counterstaining was performed with Mayer's hemalaum solution, and sections were mounted with Kaiser's gelatine (Merk Darmstadt, Germany).

Each biopsy was evaluated by two independent investigators. At least 10 high power fields (HPF) (1HPF = 0.28 mm^2) were examined at 400× magnification. Increased lymphocytic infiltration was defined as seven or more CD3-positive cells mm^2.

Analysis of the biopsies for enteroviral RNA. Enteroviral RNA was detected by RT-PCR combined with a southern blot hybridization as previously described by Pauschinger et al. (17). In brief, total RNA isolated from the biopsies was reverse transcribed into cDNA. Enteroviral specific cDNA was amplified by PCR using primers (5′Primer: 5′CGGTACCTTTGTGCGCCTGT3′; 3′Primer: 5′CAGGCCGCCAACGCAGCC3′), which are located within the 5′non-coding sequences area common for several enteroviral subtypes. Enterovirus-specific sequences were detected by southern blot analysis of the PCR products. Blots were hybridized with a 5′-P^{32} labeled oligonucleotide (5′CGAAGTAGTTGGCCGGATAAC3′), which recognizes enteroviral sequences.

Statistical analysis. Except when otherwise stated, data are shown as mean ± standard error of the mean (M ± SEM). Values were tested for normal distribution using the Shapiro-Wilk W Test. The Mann-Whitney *U* test was performed to test for statistical differences between two groups. The Krushal-Wallis analysis of variance test in ranks was used to compare more than two groups whose data were not normally distributed, and the Dunn's test was subsequently performed as a multiple-comparison procedure. Chi-square test was employed for the comparison of qualitative data. A probability value < 0.05 indicated statistical significance.

RESULTS

Patients. Fifty-three patients with clinically suspected inflammatory heart disease showing lasting symptoms over an average of 11 ± 14 months were enrolled in this study. All patients had regional or global left ventricular dysfunction as assessed by ventriculography and echocardiography. An overview of the clinical data is given in Table 1. Biopsies of 27 patients were infected with enteroviral genome. The histological investigation of the endomyocardial biopsies resulted in the diagnosis of acute myocarditis for two and borderline myocarditis for nine of the studied patients tested. Immunohistological studies detected increased intramyocardial T-lymphocyte infiltration in 25 of the enrolled patients. The duration of cardiac symptoms correlate neither with virus persistence nor with the amount of lymphocytes infiltrating the heart tissue of csIHD patients.

ANT isoform transcription pattern. The myocardial transcription pattern of the three ANT isoforms in patients with ischemic cardiomyopathy (ICM) (ANT1: 66.1 ± 1.8%; ANT2: 27.4 ± 1.5%; ANT3: 6.5 ± 0.7%; Fig. 1B) was equal to the adenine nucleotide translocator isoform transcription pattern (ANTitp) of people without any cardiac disease, as previously reported (13). In order to describe the ANT isoform pattern by the use of only one value and, therefore, simplifying the presentation of the further data, the ratio of ANT1:(ANT2 + ANT3) mRNA was calculated. It amounted to an average of 2.2 ± 0.5 (M ± SD) for the controls (Fig. 1A). The range of normal ANT isoform composition was defined as the mean value of controls ± 3 × SD (0.7–3.7). The group of patients with csIHD showed a significantly higher myocardial ANT1:(ANT2 + ANT3) mRNA ratio making up 3.6 ± 2.3 (M ± SD; $p <$ 0.02) than the controls. Twenty-one patients with csIHD exceeded the upper limit of the normal isoform ratio pointing to a shift in the ANT isoform pattern. This shift was characterized by an increase in the ANT1 mRNA (85 ± 0.9%) and a decrease in the ANT2 (11.5 ± 1%) percentage (Fig. 1B). The ANT3 mRNA proportion was only slightly lowered (3.5 ± 0.4%). The alteration in the ANTitp was not found to be correlated with the duration of the disease.

Presence of enteroviral genome. None of the control heart specimens were infiltrated with viral genome. In contrast, enteroviral RNA was detected in endomyocardial biopsies of 27 csIHD patients.

The ANT isoform mRNA pattern was analyzed in relation to the presence of enteroviral mRNA in heart tissue (Fig. 2). The average ANT1/ANT2 + ANT3 mRNA ratio of csIHD patients, found to have enteroviral genome in their myocardial tissue, was seen to be increased to 4.5 ± 0.5 and differed significantly from the controls ($p < 0.001$) and from the group of virus-negative csIHD patients ($p < 0.01$). In contrast, the myocardial ANT isoform transcription of virus-negative csIHD was not significantly distinguished from the controls (2.7 ± 0.3 vs. 2.2 ± 0.1). Enteroviral genome was found in the heart biopsies of 15 from 21 csIHD with an altered ANTitp (71.4%). In contrast, considerably less patients with a normal ANTitp were virus-positive (12/32, $p < 0.02$). Only 6 of 26 virus-negative patients (23%) were affected by a shift in the ANTitp.

Myocardial T-cell infiltration. The number of T-lymphocyte in endomyocardial biopsies taken from patients with csIHD was determined by immunohistochemical technique and was correlated with the ANT isoform transcription (Fig. 3). The maximum of normal intramyo-

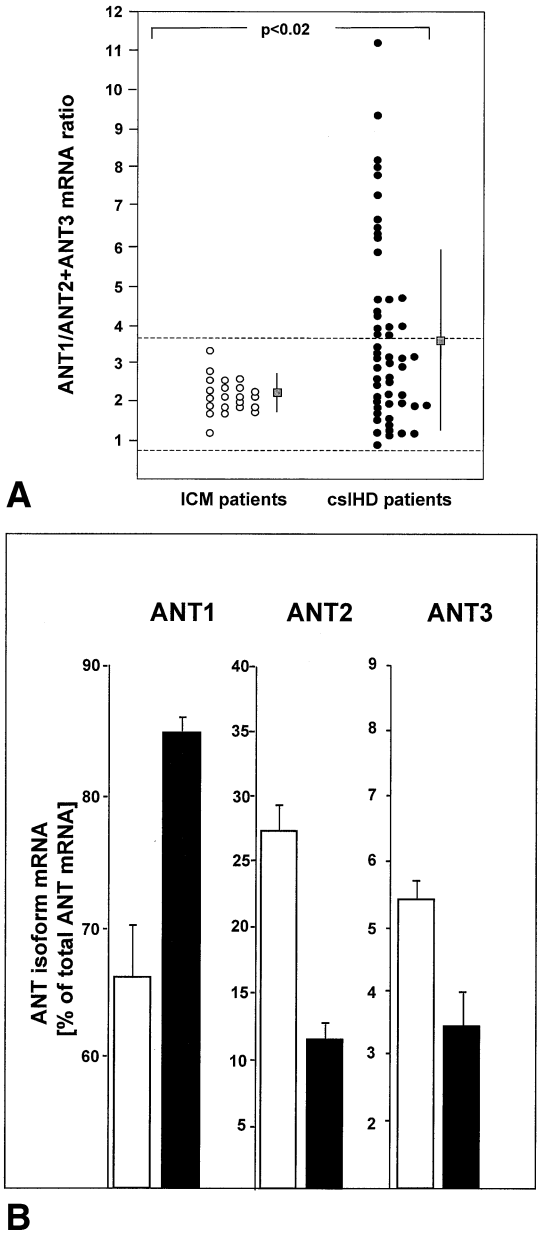

Figure 1. (A) The ANT isoform transcription pattern of patients with ICM (n = 22) and csIHD (n = 53) is described by its ANT1/(ANT2 + ANT3) mRNA ratio. Ischemic cardiomyopathy patients were found to have an identical ANT isoform profile with healthy people (13). Thus, the range of normal ANT isoform distribution was defined as the mean ± 3 SD of the ICM patient's ratio and was marked by **broken lines**. The average percentage of each ANT isoform found in the myocardium of patients with csIHD that have an ANT1/ANT2 + ANT3 mRNA ratio outside the normal range (n = 21; **solid bars**) are shown in comparison with ICM patients (n = 22; **open bars**) in graph **(B).** Data are shown as means ± SEM. ANT = adenine nucleotide translocator; csIHD = clinically suspected inflammatory heart disease; ICM = ischemic cardiomyopathy.

cardial $CD3^+$-cell amount had previously been defined to be <7 cells/mm^2 (16). An excess of this limit reveals evidence of an active T-cell dependent immune response.

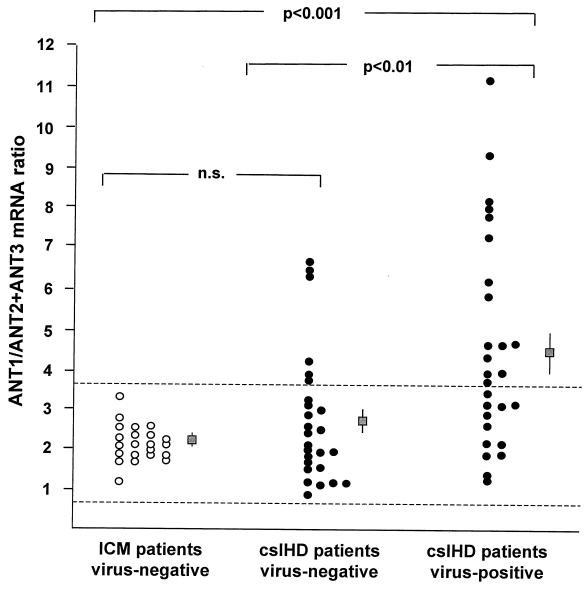

Figure 2. The ANT isoform distribution, described by the ANT1/ANT2 + ANT3) mRNA ratio, is shown for patients with ICM (n = 22) and patients with csIHD whose biopsies were free from enteroviral genome (virus-negative, n = 26) or infiltrated with enteroviral RNA (virus-positive, n ± 27). **Broken lines** mark the range of normal ANT isoform distribution. Data are shown as means ± SEM. ANT = adenine nucleotide translocator; csIHD = clinically suspected inflammatory heart disease; ICM = ischemic cardiomyopathy.

The ANT isoform shift was negatively correlated with the infiltration of the biopsies with $CD3^+$ T-cells ($p < 0.001$).

Endomyocardial specimens with a normal ANTitp were significantly higher—infiltrated with CD3-positive lymphocytes—than those with an altered isoform pattern (Table 2). Also, the amount of CD45R0-positive cells, revealing the presence of activated T-lymphocytes and T-memory cells, was remarkably higher in biopsies with an unchanged isoform. The myocardial infiltration with suppressor/cytotoxic T-lymphocytes ($CD8^+$) and inducer/helper T-cells ($CD4^+$) was found to be increased in tissue with a normal ANTitp. These differences were statistically significant for $CD8^+$ and showed a trend for $CD4^+$.

ANT isoform transcription and $CD3^+$ lymphocytic infiltration of virus-positive biopsies. As seen in Figure 2, not all of the 27 virus-positive csIHD patients showed an alteration in the ANTitp. In order to elucidate the differences between the virus-positive patients with and without an ANT isoform shift, respectively, we correlated the ANT isoform transcription profile with the intramyocardial $CD3^+$-cell infiltration of these patients (Fig. 4).

Fifteen virus-positive csIHD patients were found to be affected by the shift in the myocardial ANTitp. Only two of them showed an abnormally lymphocytic infiltration with $CD3^+$ cells in their myocardium. In contrast, 75% (9/12) of the endomyocardial biopsies from virus-positive csIHD patients with a normal ANTitp were significantly infiltrated

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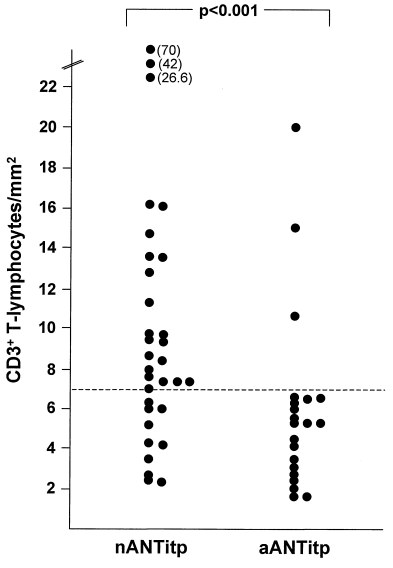

Figure 3. The amount of CD3-positive T-cells infiltrating the endomyocardial biopsies from patients with csIHD with a normal (n = 32) or an altered ANTitp (n = 21). According to Kühl et al. (16), the normal range of intramyocardial T-cell infiltration was defined to be <7 cell/mm^2. An excess of this limit, which is marked by the **broken line** in the graph, shows a pathological infiltration with T-cells. aANTitp = altered adenine nucleotide translocator isoform transcription pattern; csIHD = clinically suspected inflammatory heart disease; nANTitp = normal adenine nucleotide translocator isoform transcription pattern.

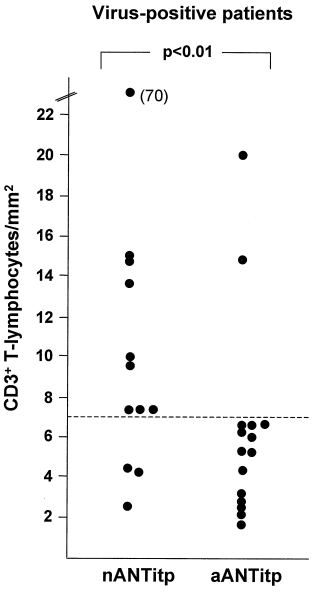

Figure 4. The amount of CD3-specific cells infiltrating the heart tissue of virus-positive patients with csIHD shown to have a normal (n = 12) or an altered ANTitp (n = 15). According to Kühl et al. (16) the normal range of intramyocardial T-cell infiltration was defined to be <7 cell/mm^2. aANTitp = altered adenine nucleotide translocator isoform transcription pattern; csIHD = clinically suspected inflammatory heart disease; nANTitp = normal adenine nucleotide translocator isoform transcription pattern.

with lymphocytes ($p < 0.01$). Consequently, most of the csIHD patients with a normal ANTitp showed a remarkable T-cell infiltration of their heart tissue, whereas an ANT isoform shift was mainly observed in virus-positive biopsies not penetrated with T-lymphocytes.

DISCUSSION

It was not until recently that interest focused on the biochemical and molecularbiological processes taking place in the enterovirus-infected myocardium (18,19). In connection with this, we recently found an alteration in the myocardial ANT expression correlated with a restricted ANT function in a significant number of patients suffering from myocarditis and DCM (5). This study was designed to enhance our understanding of the conjunction between the enteroviral infection, the T-cell dependent immune response and the shift in the ANT isoform transcription in human inflammatory heart disease.

Table 2. Amount of T-lymphocyte Subtypes Infiltrating the Biopsies of Patients With a Normal or an Altered ANT Isoform Transcription Pattern

T-Cell Subtype	nANTitp	aANTitp	p Value
CD3$^+$ (cells/mm^2)	12.0 ± 2.3	6.1 ± 1.0	<0.01
CD45R0$^+$ (cells/mm^2)	7.1 ± 2.2	2.2 ± 0.5	<0.01
CD8$^+$ (cells/mm^2)	8.2 ± 2.2	3.1 ± 0.2	<0.01
CD4$^+$ (cells/mm^2)	5.5 ± 2.1	2.5 ± 0.4	n.s.

aANTitp = altered adenine nucleotide translocator isoform pattern; nANTitp = normal adenine nucleotide translocator isoform pattern; n.s. = not statistically significant.

A myocardial altered ANT isoform mRNA pattern is linked to an enteroviral infection. Acute myocarditis induced by an enteroviral infection occurs during the first two weeks. Within this time myocytolysis and virus clearance take place. Depending on the genetic background of the patients or the state of their immune system, myocarditis either disappears spontaneously within the next four months or becomes chronic. In the chronic phase, virus persistence or the amount of infiltrating immunocompetent cells was found to be independent of the duration of the disease. These findings were confirmed in this study. The interval between the onset of cardiac symptoms and biopsy was not seen to be related either to the level of T-cell infiltration or to the enteroviral infection or to the ANT isoform shift. Therefore, data could be correlated independently from the anamnestic onset of the disease.

Twenty-one of 53 patients with clinically suspected inflammatory heart disease were found to be affected by a shift in the ANT isoform pattern. The altered ANT isoform profile was characterized by an increased ANT1, a decreased ANT2 and a slightly lowered ANT3 mRNA proportion.

We found a close relation between the presence of enteroviral RNA in the heart tissue and the shift in the ANT isoform transcription. Seventy-one percent of the

biopsies from csIHD patients with an altered myocardial ANT isoform profile were infected with enteroviral RNA. The connection between the changes in the ANT function and expression and an enteroviral infection was confirmed by our studies of Coxsackie B3 infected A/J mice (14). The infection led to an increase in the intramitochondrial ATP concentration and an accumulation of ADP in the cytosol of the heart. This kind of imbalance in the intracellular adenine nucleotide distribution is typical if the activity of the ANT is impaired. Similar to human ischemic cardiomyopathy, there was no alteration in the ANT function in ischemic hearts of guinea pigs (20) or of spontaneous hypertensive rats (unpublished data), making these changes specific for an enterovirus-induced heart disease.

A few enterovirus-negative patients were also affected by the ANT isoform shift. There may be subtypes of enterovirus that were not identified by the technique used but are able to influence the ANT isoform expression. In addition, it has been shown that DNA virus, such as adenovirus (21) of HIV virus (22), were found in inflamed human heart tissue. The frequency of an adenoviral infection in patients with clinically suspected myocarditis was found to be similar to that of an enteroviral infection (21). Further studies are required to clarify whether such viruses have a similar capacity to induce an ANT isoform shift, indicating a common mechanism in causing myocardial inflammation.

The ANT isoform shift is associated with an inactive T-cell immune response. Studying the intracardial infiltration with immunocompetent cells in csIHD patients, we found a remarkable inverse correlation between the intramyocardial infiltration with T-lymphocytes and the alteration in the ANTitp. Heart tissue with a normal isoform transcription was remarkably more penetrated by active T-lymphocytes than myocardial tissue with an altered ANT isoform distribution. An ANT isoform shift occurs especially in such tissue that is infected with enterovirus but less infiltrated with T-lymphocytes. We, therefore, conclude that an active cellular immune response produces conditions that prevent the ANT isoform shift. This rescuing effect may be a result of the elimination of the virus from the myocardium. Subtyping the T-lymphocytes, infiltrating the analyzed human biopsies, a significant increase in the infiltration with ($CD8^+$) T-lymphocytes was found in the heart tissue with a normal ANT isoform profile. In contrast with this, the amount of $CD4^+$ cells was only somewhat elevated. The relevance of cytotoxic/suppressor ($CD8^+$) lymphocytes for the clearance of the virus from the tissue has been demonstrated in murin myocarditis models and especially in Coxsackie B3 infected CD8-knockout mice (23,24). The exclusion of $CD8^+$ lymphocytes from the cellular immune system resulted in an enormous increase in the virus titer in the myocardium of the animals.

Mechanisms that might be responsible for the ANT isoform shift. Even if the association between the enteroviral infection and the change in the ANT expression is evident, the mechanisms responsible for this alteration remain unknown. Low-level CB3 gene expression without a formation of infectious virus progeny was found to induce a cytopathic effect in transfected myocytes, which was demonstrated by a release of lactate dehydrogenase from transfected myocytes (25). These findings support the opinion that the virus is directly involved in the alteration in the host's gene expression. Other studies underline the assumption that virus-induced autoimmunologic processes influence myocardial energy metabolism by depressing the ANT function (26). Moreover, enterovirus stimulate the production of cytokines and inflammatory mediators, such as NO, which themselves induce changes in the biochemical and molecular biological processes of myocytes. In the mouse model of chronic viral myocarditis, a progressive expression of IL1 beta, IL6, TNFa was shown when most histological signs of inflammation had subsided (27). Cytokines and NO were shown to cause a time-dependent induction of cardiac myocyte apoptosis (28) in which the ANT plays an important role (7). Such an elevated expression of cytokines, such as TNF alpha, IL1 beta, IL6, IL8 and NO was also observed in the myocardium of patients with myocarditis and DCM (29–31).

Consequences of an altered ANT function and expression for the heart. Previous studies have demonstrated that the shift in the transcription pattern is also seen on the protein level shown by an increase in the ANT1 and the total ANT protein amount (7). The change in the ANT protein amount was accompanied by a decrease in the transport capacity of the ANT (12). A disturbed energy metabolism and an impaired heart function was shown to be a consequence of an altered ANT expression and function in animal models, such as ANT1 knockout mice (32) and Coxsackie B3 infected mice (14). In these cases myocardial dysfunction was a result of a lowered ATP supply. However, the ANT is also highly involved in the apoptosis process (33). It is a member of the permeability transition pore and an important receptor for apoptosis-regulating proteins of the Bcl-2 protein family (7). Thus, changed ANT expression might not only influence the energy metabolism but also the apoptotic process. Apoptosis was seen to be active in enterovirus infected heart tissue (34) and is supposed to play a significant role in human heart failure.

Other genes, coding for proteins that are involved in apoptosis, signal transduction pathway, regulation of gene expression and oxidative phosphorylation were also found to be differently expressed in Coxsackie B3-infected mice (35). The altered ANT gene expression thus appeared to be a feature of a specific gene program activated in enterovirus-infected hearts.

The data led to the conclusion that the virus itself, or factors that are activated by virus-infection, influence the ANT isoform expression in heart tissue, which show less or no evidence of an active T-cell dependent immune response. In view of the significance of the ANT for complex

intracellular processes, such as the energy metabolism and apoptosis, alterations in the ANT expression and function may be involved in the pathophysiology of viral heart disease.

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