Antibodies to the ADP/ATP Carrier, an Autoantigen in Myocarditis and Dilated Cardiomyopathy, Penetrate Into Myocardial Cells and Disturb Energy Metabolism In Vivo

Karsten Schulze, Bernhard F. Becker, and Heinz P. Schultheiss

We identified the ADP/ATP carrier, located within the inner mitochondrial membrane, to be an organ- and conformation-specific autoantigen in myocarditis and dilated cardiomyopathy. We also showed that autoantibodies to the ADP/ATP carrier inhibit the nucleotide transport in vitro. Specific binding of the autoantibodies to the carrier was demonstrated by radioimmunoassay and the immunoblot technique; the inhibition of the nucleotide transport was determined by the inhibitor stop method. To establish if these autoantibodies might also affect cardiac energy metabolism in vivo, we measured whether they are capable of penetrating into myocytes and whether subcellular ATP/ADP ratios and phosphorylation potentials of ATP change in hearts of guinea pigs that have been immunized with the isolated ADP/ATP carrier. An intracellular deposition of autoantibodies was observed by direct immunofluorescence and by immunoperoxidase staining on cryosections of the myocardial tissue of animals immunized with the ADP/ATP carrier. Furthermore, binding of autoantibodies to mitochondrial membrane structures was shown by immunoelectron-microscopic methods. The cytosolic and intramitochondrial distribution of adenine nucleotides in stimulated, isolated perfused hearts of guinea pigs immunized with the ADP/ATP carrier was measured by nonaqueous fractionation. Compared with controls performing equal external heart work, the cytosolic ATP decreased in the immunized animals, whereas the mitochondrial ATP increased strongly; ADP concentrations showed an opposite change. Thus, a resultant cytosolic decrease and a marked mitochondrial increase of the ATP/ADP ratio was established. As a consequence, the cytosolic-mitochondrial phosphorylation potential of ATP was diminished. These findings demonstrate that antibodies against intracellular antigens are able to penetrate into living cells, and that autoimmunity to the ADP/ATP carrier may contribute to the pathophysiology of myocarditis and dilated cardiomyopathy by causing an autoantibody-mediated imbalance between intracellular energy delivery and demand. (Circulation Research 1989;64:179-192)

Current evidence indicates that immunological mechanisms are involved in the pathogenesis of myocardial damage in dilated cardiomyopathy deriving from myocarditis.1-4 Many patients with acute myocarditis later suffer from chronic dilated cardiomyopathy.5 Conversely, in many cases dilated cardiomyopathy appears to represent a sequel to myocarditis, though myocarditis may already be inactivated or continuing as a smoldering process.5 Indeed, virus persistence has been observed in the hearts of patients with dilated cardiomyopathy who previously suffered from myocarditis. Thus, the virus infection may initiate an autoimmune process through antigenic determinants shared by the virus and the host cell or by altering the host's immune system, thereby causing the release or the expression of sequestered antigens. No reports on a possible role of autoantigens in myocarditis and dilated cardiomyopathy exist to date. However, we recently showed that patients with these diseases have autoantibodies against the ADP/ATP carrier, a protein in the inner mitochondrial membrane.8-10 This transport protein is of great importance for the energy metabolism of the eukaryotic cell.11 It is responsible for both the

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“import” of cytosolic ADP into the mitochondrial matrix space for rephosphorylation and the “export” of ATP for the energy-consuming reactions of the cytosol, because the inner mitochondrial membrane is impermeable to the hydrophilic nucleotides ADP and ATP.

On further characterization, these autoantibodies turned out to be organ- and conformation-specific. This means that they react mainly with the ADP/ATP carrier of the heart and to a much smaller extent with, for example, the liver or the kidney protein. Furthermore, binding of the autoantibodies takes place only when the carrier is in the “c-conformation,” with the substrate binding site directed to the cytosol. In addition, the autoantibodies were shown to inhibit the nucleotide exchange in vitro by blocking the substrate binding site of the transport protein.

Recent data indicate that inactivation of autoantibodies against the ADP/ATP carrier may be a useful marker for the identification of persons who are at greater risk for the development of dilated cardiomyopathy. These findings raise the question whether these antibodies are able to influence the function of the carrier protein even in vivo, or whether their appearance in myocarditis and dilated cardiomyopathy is only an epiphenomenon. The aim of our investigations was to answer this question.

Since no methods are available for measurement of the function of the ADP/ATP carrier in the living organ, the difference of the phosphorylation potential of ATP between the cytosol and the mitochondria was determined to reveal any antibody-mediated loss of the transport activity of the carrier. In our experimental model, we applied the method of nonaqueous fractionation to yield the subcellular values of the phosphorylation potential in spontaneously beating, working heart preparations isolated from immunized guinea pigs. The present study shows that antibodies against the ADP/ATP carrier cause an antibody-mediated imbalance between energy delivery and demand. On the basis of these data one can suggest that, at the molecular level, such antibodies may be mediators of autoimmune diseases, and that dilated cardiomyopathy may be one of these.

Materials and Methods

Isolation of the ADP/ATP Carrier

Beef heart mitochondria were isolated as described by Smith. The solubilization and isolation of the ADP/ATP carrier followed the procedure previously described. In brief, mitochondria were first loaded with the tightly binding and specific inhibitor carboxyatractylate (CAT). The suspension of mitochondria (10 mg protein/ml in 250 mM sucrose, 10 mM Tris-HCl, 50 mM ADP, pH 7.2) was supplemented with 3H-CAT (4 mmol/g protein, specific activity 4,000 cpmmol), left standing for 30 minutes at 4°C, and then centrifuged for 30 minutes at 10,000g. The protein was thus fixed in the c-conformation, that is, facing the cytosolic side of the membrane, and was protected against detergent-induced denaturation. The 3H-CAT binding also served as a marker during the isolation procedure. The pellet was subsequently treated with the detergent Triton X-100 (Sigma, Munich; concentration, 6% wt/vol).

After solubilization and centrifugation (30 minutes at 140,000g) the supernatant was applied to a hydroxyapatite column (4 mg protein/ml column volume), which had been equilibrated with a solution of 0.1 M NaCl, 0.5% Triton X-100, and 10 mM 2-(N-morpholino)-propane sulfonic acid (MOPS) at pH 7.2. The protein was eluted at 4°C with the same buffer. The enriched carrier protein (assessed from the CAT/protein ratio) was pooled and applied to a gel chromatography column (6×100 cm; Ultrogel ACA 34, LKB, Uppsala, Sweden). The protein was eluted with a buffer consisting of 175 mM Na2SO4, 10 mM MOPS, and 0.5% Triton X-100 (pH 7.2). To remove minor contaminants, excess Triton X-100, and phospholipids from the protein, the eluant was finally subjected to sucrose density gradient centrifugation with a linear gradient range of 5–20%. The total enrichment was about 10-fold.

Immunoassay Procedure

Blood was taken from guinea pigs 1 week before immunization to obtain preimmune sera. Fifteen female guinea pigs (1,000 g; Firbright, Ivanovas, Munich) were immunized with the ADP/ATP carrier isolated from beef heart mitochondria. For immunization, carrier protein was injected both subcutaneously (30 µg) and intraperitoneally (20 µg) every second week for 4 months. The CAT-protein complex (0.1 mg protein suspended in 100 mM NaCl, 10 mM MOPS, and 0.5% Triton X-100) was emulsified with an equal volume of Freund’s complete adjuvant. The booster injections were administered subcutaneously in Freund’s incomplete adjuvant. The antigen injected intraperitoneally was given without Freund’s adjuvant.

Isolation of Immunoglobulins

Immunoglobulin (IgG) was fractionated using protein-A Sepharose in 10 mM phosphate-buffered saline (PBS), pH 7.4, containing 0.1% sodium azide. The column (0.9×15 cm) was stored at 4°C. After the serum was added, the column was washed with PBS until the light absorbance of the eluate at 280 nm was at background level. Immunoglobulin was then eluted with 0.58% (wt/vol) glacial acetic acid in 0.15 M NaCl (pH 2.8). After neutralization of the eluate with 0.2 M Tris, the IgGs were dialyzed against PBS.

Indirect Micro Solid-Phase Radioimmunoassay (SPRIA)

A modified SPRIA was performed on flexible, U-shaped polystyrene microtiter plates (Dynatech Sci...
ADP/ATP carrier was diluted to the optimal concentration (0.5 mg/ml) in a solution of 100 mM Na₂SO₄, 10 mM MOPS, and 0.5% Triton X-100 (pH 7.2), and a 100-μl aliquot was incubated at 4°C for 4 hours. After three washings with 3% fetal calf serum in PBS, the precoated plates were incubated with 100 μl of a filler (3% fetal calf serum) for 1 hour at 4°C to block any remaining active binding sites of the polyvinyl plate. After a further washing, the antigen-coated wells were incubated overnight at 4°C with PBS, the precoated plates were incubated with 100 μl of a filler (3% fetal calf serum) for 1 hour at 4°C to block any remaining active binding sites of the polyvinyl plate. After a further washing, the antigen-coated wells were incubated overnight at 4°C with 90 μl of the serum to be tested. Subsequent to renewed washing, specifically bound antibodies were detected with iodinated protein A (100,000 cpm/well; New England Nuclear, Boston, Massachusetts). After incubation (4 hours at 4°C), the wells were washed three times, allowed to dry, and counted in a gamma spectrometer. All assays were performed twice. The separate control for nonspecific binding, performed parallel to each test with a "no-antigen" (filler only), a "no-serum," and a "control-serum," routinely gave 2–6% of the total activity.

**Western Blot**

The isolated ADP/ATP carrier, the total protein from heart mitochondria, and marker proteins were separated on sodium dodecyl sulfate polyacrylamide slab gels. One section of the gel was stained with Coomassie blue; the other section was electrophoretically blotted on a wet nitrocellulose sheet (0.45 mm; Bio Rad, Munich, FRG), sandwiched between filter paper and scouring pads supported on a porous plastic grid. The running buffer, pH 8.3, contained 25 mM Tris, 200 mM glycine, and 30% methanol. The electrophoretic transfer was run overnight at 4°C with 0.2 A. The nitrocellulose was then placed for 2 hours in a buffer of 50 mM Tris/HCl (pH 7.4) and 150 mM NaCl (TBS) supplemented with 2.5% bovine serum albumin (BSA). Afterwards, it was incubated for 2 hours with guinea pig antiserum in TBS with 2.5% BSA. To remove unbound antibodies, six washings of 5 minutes each were performed with TBS that did not contain BSA. Those antibodies that remained bound to the nitrocellulose were stained by horseradish peroxidase conjugated anti-IgG.

**In Vitro Measurement of Adenine Nucleotide Transport**

The activity of the antibodies with respect to inhibition of nucleotide transport was tested in vitro on isolated mitochondria by measuring the exchange of ³⁵S-labeled ADP with the inhibitor stop method. Isolation of mitochondria from guinea pig hearts was performed according to Smith. Batches of isolated mitochondria (20 mg) were next loaded with 0.8 μCi ³⁵S-ADP each. The ADP exchange was started by adding 10 μl of unlabeled ADP (10 mM) to 200 μl of suspended mitochondria. After 5, 10, 20, and 40 seconds, the ADP transport was stopped by the addition of carboxyatractylate. The adenine nucleotide translocation, a 1:1 exchange between intramitochondrial and extramitochondrial nucleotides, may be expressed as a percentage exchange of the total intramitochondrial content and calculated according to the equation:

\[
\text{percentage exchange} = \frac{\text{cpm (assay)} - \text{cpm (control)}}{\text{cpm (total)} - \text{cpm (control)}} \times 100
\]

**Working Heart Preparation**

The hearts of the 10 guinea pigs with the highest serum antibody titers were isolated and perfused as working heart preparations, as described by Becker et al and Bünger et al. The nonrecirculating perfusion medium was a modified Krebs-Henseleit buffer containing (mM) NaCl 127, KCl 4.7, NaHCO₃ 24.9, CaCl₂ 1.25, MgSO₄ 0.6, KH₂PO₄ 1.2, enriched with pyruvate 0.3 mM, glucose 5.5 mM, and insulin 5 U/l, and equilibrated at 37°C with 94.4% O₂-5.6% CO₂ (pH 7.4).

Perfusate was applied via a cannula tied into the left atrium; all other atrial openings were ligated. The preload of 12 cm H₂O and the afterload of 80 cm H₂O (mean developed pressure) determined the pressure-volume-work of the left ventricle. No external work was performed by the right ventricle. The right atrial veins were ligated, and the coronary venous effluent was drained through a cannula inserted in the pulmonary artery.

Atrial filling pressure and aortic pressure were monitored by Statham P23BB and P23Db strain gauges (Gould, Cleveland, Ohio). Heart rate was derived from the phasic pressure signals with a Beckman cardiotachometer (Fullerton, California). All parameters were recorded on a Beckman Dynograph R411. External heart work was calculated as the sum of pressure-volume-work and acceleration work during ejection. The following formula was applied:

\[
\text{External heart work} = [(\text{cardiac output}) \times (\text{pressure gradient across the left ventricle})] + \frac{1}{2} \times (\text{ejected volume}) \times (\text{mean velocity of flow})^2
\]

The myocardial oxygen consumption was derived from the difference of O₂ tension between aortic perfusate and coronary effluent, measured with two Clarke-type electrodes (Bachofer, Reutlingen, FRG). The lactate released into the coronary effluent was measured enzymatically.

To reach a comparable activation of energy metabolism, all hearts (control and immunized groups) were equally stimulated with norepinephrine. It was not intended to reach the maximal stimulation level for each heart because of the temporal instability and, thus, the ill-defined perfusion conditions in such states. After stabilization of the working heart prep-
arations (approximately 30 minutes), 0.08 µM nor-
epinephrine was infused into the left atrial cannula
by a Precidor pump (Infors, Basel, Switzerland). The
infusion lasted about 20 minutes, allowing the hearts
to attain a steady state of external heart work and
oxygen consumption. In this condition the hearts
were clamped between a pair of aluminum tongs that
had been precooled in liquid nitrogen.

Nonaqueous Fractionation
The frozen hearts were pulverized in a mortar
filled with liquid nitrogen. To avoid further meta-
blogic processes, the crushed hearts were lyo-
philized at -40° C, 0.001 mm Hg, and then stored in
heptane/carbon tetrachloride. During lyophilization
all formerly dissolved substances (enzymes and metabo-
lites) cling to the membrane walls of the
respective cellular compartment. After ultrasonic
disruption of the cells into small membrane frag-
ments, insufficiently homogenized particles, consist-
ing mainly of connective tissue, were removed by
successive filtration through columns filled with
glass beads of 1.0 and 0.4 mm diameter. The puri-
fied homogenate was then subjected to density
gradient centrifugation (4 hours at 16,000g). The
heptane/carbon tetrachloride density gradients (1.29–
1.38 g/ml) were produced by a continuous variation
of the volume ratio of the heavier carbon tetrachlo-
ride (1.59 g/ml) and the lighter heptane (0.69 g/ml).

Since mitochondrial membranes are lighter than
cytosolic structures, fractions with different propor-
tions of cytosolic and mitochondrial protein can be
obtained after density gradient centrifugation.25 Each
of these six to eight fractions was divided into two
aliquots. In one aliquot, the activities of the cyto-
solic marker enzyme phosphoglycerate kinase (PGK)
and the mitochondrial marker enzyme citrate syn-
thase (CS) were determined as well as the total
protein contents. In the second aliquot, the con-
tents of phosphate, ADP, ATP, creatine, and phos-
phocreatine were analyzed. Protein was measured
according to the method of Lowry26; all other tests
were enzymatic analyses.27-28

Calculation of Intracellular Metabolite
Concentrations
The known total content of a metabolite (M) in
each fraction of the density gradient is the sum of its
mitochondrial and cytosolic portions. Thus,

\[ M_{tot} = M_{cyt} + M_{mil} \]  \hspace{1cm} (1)

In addition, the activities of the marker enzymes
PGK and CS, determined in each fraction, correlate
with the membrane content and the metabolite
content of the respective cellular compartment.
This relation between marker enzyme and compart-
mental metabolite content is constant in all fractions
of the gradient and is expressed as

\[ M_{cyt}/PGK = a \]  \hspace{1cm} (2)

\[ M_{mil}/CS = b \]  \hspace{1cm} (3)

Combination of Equations 1, 2, and 3 and division
by CS leads to the linear expression

\[ M_{mil}/CS = a (PGK/CS) + b \]

The factors \( a \) and \( b \) can be readily obtained by linear
regression, whereupon the cytosolic and mitochon-
drial metabolite contents can be calculated from
Equations 2 and 3. To derive concentrations these
contents were first referred to the protein contents of
the corresponding compartment. Then, subcellular
concentrations were obtained assuming 3.8 µl water/
mg cytosolic protein and 1.8 µl water/mg protein in
heart mitochondria.29-30 Since most of the cytosolic
ADP is bound to the contractile system of the heart
muscle, the free cytosolic ADP was calculated from
the creatine phosphokinase reaction, assuming an
intracellular hydrogen concentration of pH 7.05 and
an equilibrium constant of \( K_{CPK} = 2.04 \times 10^{-9731-32} \):

\[ [ATP]/[ADP] = [phosphocreatine] \cdot [H^+] / ([creatine] \cdot K_{CPK}) \]

As a working measure of the phosphorylation
potential of ATP \( \Delta G_{ATP} = \Delta G_0 + RT \cdot \ln([ATP]/
[ADP] \cdot [phosphate]) \), just the concentration term
of the potential was calculated; that is, \( \Delta G' =
RT \cdot \ln([ATP]/([ADP] \cdot [phosphate])) \), where \( R \)
represents Faraday's constant and \( T \) the absolute
temperature.

Statistical Analysis
The Student's \( t \) test for unpaired samples was
used. Values are given as mean±SD.

Direct Immunofluorescence
Just before freeze-clamping (see above), a small
part of the guinea pig hearts was fixed in 2-
methylbutane and stored in liquid nitrogen. Cryo-
sections (4 µm maximum thickness) of the myocar-
dial tissue were washed with PBS for 30 minutes
(KH2PO4 5.45 g/l, Na2HPO4 1.662 g/l, NaCl 7.01 g/
l, NaN3 0.4 g/l, pH 7.2). Sections were incubated
for 30 minutes with a fluorescein-isothiocyanate
conjugated plasma protein antiserum (rabbit anti-
guinea pig IgG, Behring-Institute, Marburg, FRG)
in a 1:100 PBS dilution. After three 30-minute
washings in PBS, the sections were covered with a
drop of glycerine for fluorescence microscopy. Pre-
imune serum was used as control.33

 Peroxidase Staining
Cryostat sections of the myocardium of the immu-
nized guinea pigs were air-dried for 60 minutes and
then fixed in acetone (10 minutes). A modification
of the unlabeled antibody enzyme method34 was
next applied:

1. Negative controls were performed by incubat-
ing slices with excess sheep anti-guinea pig immu-
noglobulin serum or with normal sheep globulin.
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FIGURE 1. Left panel: Binding of antibodies to isolated ADP/ATP carrier in solid-phase radioimmunoassay from sera of guinea pigs immunized with ADP/ATP carrier. Values for radioactivity after incubation with ¹²⁵I-labeled protein A are experimental results minus background. Right panel: Inhibition of nucleotide transport assessed in vitro in sera of guinea pigs immunized with ADP/ATP carrier by inhibition of ¹⁴C-ADP exchange of isolated mitochondria after 5, 10, 20, and 40 seconds. ●, control (n=5); △, immunized animals without inhibition of ¹⁴C-ADP exchange (n=4); ○, immunized animals with inhibition of ¹⁴C-ADP exchange (n=6).

Sheep anti-guinea pig immunoglobulin serum was prepared by injecting sheep subcutaneously with 5 mg of guinea pig IgG and with Freund's complete adjuvant. Seven days after the last of three booster injections applied at 14-day intervals, the animals were exsanguinated.

2. The sections were incubated with peroxidase-antiperoxidase complex. Each incubation lasted 60 minutes at room temperature and was terminated with amino-ethyl-carbazol. The sections were counterstained with hemalum. The absence of nonspecific reactions was demonstrated in heart sections of normal rabbits.

Results

Of the 15 guinea pigs immunized with the isolated ADP/ATP carrier, 10 animals showed a marked increase of antibody titer and were consequently selected for further testing. Titer heights of these 10 animals are compared with control values of five animals in Figure 1. The rise of antibodies against the carrier protein was proved by solid-phase radioimmunoassay. The specificity of the antibodies for the translocator protein was further demonstrated by Western blot technique. When the SDS gels of mitochondria or the isolated carrier protein was blotted on nitrocellulose paper, the antibodies reacted only with the transport protein (Figure 2).

The back-exchange of ADP was measured by the inhibitor stop method using isolated mitochondria loaded with ¹⁴C-ADP and incubated with the purified immunoglobulins of the sera of these 10 selected animals. In six cases a significant inhibition of the nucleotide transport was observed at 5, 10, 20, and 40 seconds (Figure 1). Thus, an antibody-mediated inactivation of the carrier function in vitro could be expected. The sera of the remaining four animals exhibited an antibody titer but had no effect on mitochondrial ADP transport.

Immunohistochemical studies by direct immunofluorescence and immunoperoxidase staining of the hearts of the immunized animals showed an intracellular deposition of IgGs that was lacking in controls (Figures 3–5). It was possible to confirm the intracellular deposition of antibodies by peroxidase-antiperoxidase electron microscopy; Figure 5 shows a binding of such IgGs to mitochondrial membranes. No staining of mitochondrial membrane structures was observed in slices of control hearts.

The hearts of five control animals and of the 10 immunized animals that exhibited the highest rate of production of antibodies against the ADP/ATP carrier were isolated and perfused as preparations performing pressure-volume-work. All hearts were stimulated by the same dose of norepinephrine to increase external work performance and enhance energy metabolism. As shown in Table 1, left ventricular external heart work performed by the hearts of immunized and nonimmunized animals did not differ. Also, the oxygen consumption of the myocardium was similar for control hearts (11.9 μmol/[g·min]) and for the hearts of the immunized animals (11.5 μmol/[g·min]). Thus, metabolic stim-
FIGURE 2. Western blot for detection of antibodies against ADP/ATP carrier of hearts from immunized guinea pigs. 1-3: SDS polyacrylamide slab gels stained with Coomassie blue. 1, marker proteins (molecular weight in daltons); 2, isolated ADP/ATP transport protein (32,000 daltons); 3, total heart mitochondrial proteins. 4—5: Nitrocellulose blots, incubated successively with serum of immunized guinea pigs and then with horseradish peroxidase conjugated anti-IgG. 4, isolated ADP/ATP transport protein; 5, total heart mitochondrial proteins.

ulation and, consequently, activation of oxidative phosphorylation can be assumed to be identical in all hearts tested.

The lactate release in the coronary effluent was measured as an expression of the rate of anaerobic glycolysis. Myocardial lactate production reached 0.67 /μmol/(g • min) in the control hearts and tended to be higher in the hearts of the immunized animals, where 0.90 /μmol/(g • min) was measured.

More direct data concerning the energy metabolism of the tested hearts were obtained by nonaqueous fractionation of the freeze-clamped hearts. Application of this technique allowed the separate evaluation of both the cytosolic and mitochondrial ATP and ADP concentrations in the myocardial cells just before clamping. These results are demonstrated in Table 2 and Figures 6-9. On the basis of the ADP-transport studies described above, we divided the immunized animals into two subgroups. The first group of hearts comprised the six guinea pigs that showed an antibody-mediated effect in the in vitro testing. These hearts indeed proved to have the most striking differences in subcellular nucleotide concentrations. The second group contained the four animals without evidence of altered function of the transport protein in vitro. The subcellular nucleotide concentrations of these hearts closely resembled the control values.

Therefore, all results described subsequently will refer to the first subcollective of six immunized guinea pig hearts.

The most striking changes were found inside the mitochondria, especially with respect to ATP (Figure 8). Two hearts reached a mitochondrial ATP of about 25 mM, three times the ATP of the controls (8.1±2.3 mM). The other four hearts had a mitochondrial ATP of 11–17 mM, still well above the control value. Because the mitochondrial ADP concentrations showed a change opposite to that of ATP, the ATP/ADP ratios revealed even greater differences (Figure 8). This quotient was approximately 5.1±0.5 in the control hearts but climbed to 17.1±4.9 (p<0.005) in the hearts of the animals that had generated inhibitory antibodies.

The cytosolic variations were less distinct. However, the cytosolic ATP/ADP ratio of 50.2 was significantly lower than the control mean value of 61.8 (p<0.05) (Figure 8). In two of the hearts with mitochondrial nucleotide shifts, quite low levels of ATP also were measured in the cytosol (8.8 mM and 7.9 mM versus control 13.2±2.0 mM) and phosphocreatine decreased from 26.1 mM (control) to 13.2 mM and 20.5 mM.

The cytosolic phosphorylation potential (ΔG'_{ATP}) decreased from 22.2±0.4 kJ/mol ATP (control) to 21.4±0.3 kJ/mol ATP (p<0.005), but the difference was not as pronounced as the increase of the mitochondrial phosphorylation potential, which rose from 17.6±0.6 kJ/mol ATP (control) to 20.7±1.2 kJ/mol ATP (p>0.005).

The cytosolic-mitochondrial difference of ΔG'_{ATP} expresses best the state of the energy metabolism of the myocardial cell, and its value combines all of the variables evaluated. Figure 9 visualizes the decrease of ΔG'_{(cyt-mito)} from 4.6 kJ/mol ATP in controls to 0.7 kJ/mol ATP (p<0.005) in the hearts of the six immunized animals.

Discussion

The decisive result of our experiments is the significant shift of intracellular nucleotide concentrations in hearts of six guinea pigs that, after immunization with the isolated ADP/ATP carrier, generated carrier-inhibiting antibodies. These altered nucleotide concentrations caused a pronounced drop in the cytosolic-mitochondrial difference of the phosphorylation potential of ATP. Compared with five controls, the cytosolic ATP concentration decreased from 13.2 mM to 11.3 mM. The mitochondrial ATP concentration, however, increased from 8.1 mM to 18.3 mM (p<0.005). The cytosolic-mitochondrial difference of the phosphorylation potential of ATP was diminished from the control value of 4.6 kJ/mol ATP to 0.7 kJ/mol ATP (p<0.005). It is of interest that the measured control values of nucleotide concentrations and phosphorylation potentials are in good agreement with those reported by Soboll25 and Geisbuhler.36
Marked changes in high-energy phosphates have not been observed so far in the myocardium of patients with heart failure. This unaltered global content of high-energy phosphates could simply reflect a compensation of the cytosolic and mitochondrial changes. However, in patients with cardiomyopathy, an impaired mitochondrial function and an activation of compensatory mechanisms, such as increased activity of lactate dehydrogenase, have been demonstrated. Furthermore, recent studies by our group revealed a decreased activity of the ADP/ATP carrier in 10 of 20 patients with dilated cardiomyopathy.

In general, the observed diminished difference of the cytosolic-mitochondrial phosphorylation potential of ATP can be caused either by a reduction of oxidative phosphorylation, resulting in a lowered potential over the inner mitochondrial membrane, by a lowered ATP-synthase activity, or by an inactivation of the ADP/ATP carrier. A reduction of the mitochondrial membrane potential cannot be totally excluded, for we did not measure it directly. However, the hearts of the immunized guinea pigs exhibited an oxygen consumption equal to that of the control hearts. Because this is an indirect parameter of the function of oxidative phosphorylation, the respiratory chain per se seems not to be influenced by the induced immunological processes. Consequently, it can be assumed that the electrochemical gradient over the inner mitochondrial membrane does not differ between the myocardial cells of immunized and nonimmunized animals. More-
over, ATP synthesis does not appear to be affected. This can be concluded from the high mitochondrial ATP concentrations in the immunized hearts.

The imbalance between an apparently unchanged high electrochemical gradient at the inner mitochondrial membrane and the observed low cytosolic-mitochondrial difference of the phosphorylation potential of ATP is an adequate stimulus for the ADP/ATP carrier to give preference to an asymmetric mitochondrial ATP efflux and ADP influx. \textsuperscript{42,43}

The nucleotide exchange is driven by the mitochondrial transmembrane potential. \textsuperscript{44} The positively charged outside of the mitochondrial membrane favors extrusion of the negatively charged ATP. On the other hand, ADP, bearing one negative charge less than ATP, can be taken up into the matrix space more easily. \textsuperscript{45-47} The size of the membrane potential and the ratio of the transmembrane ADP versus ATP net-exchange correlate directly.

Under physiological conditions this kind of asymmetric transport creates high cytosolic and low mitochondrial ATP/ADP ratios. \textsuperscript{49} Consequently, the phosphorylation potential of ATP, the variable decisive for the energy state of the cell, is elevated in the cytosol and lowered in mitochondria. \textsuperscript{23,43}

During functional stimulation of the hearts, a lowered cytosolic $\Delta G'_{\text{ATP}}$ was measured,\textsuperscript{25} reflecting ATP consumption through heightened metabolic demand. This finding suggested the ADP/ATP carrier to be one of the assumed rate-controlling steps in oxidative phosphorylation. \textsuperscript{48} The decrease of the cytosolic-mitochondrial difference of $\Delta G'_{\text{ATP}}$ stimu-
lates the ADP/ATP carrier to a higher asymmetry in nucleotide transport so as to supply more ATP into the cytosol. Asymmetric ATP-ADP transport continues until the energy balance between the transmembrane potential and $\Delta G'_{(ctt-mito)}$ is restored. Under normal conditions of cardiac load, the nucleotide exchange capacity of the carrier is obviously high enough to still guarantee a stable intracellular nucleotide distribution.

In nonimmunized animals the ADP/ATP carrier is near equilibrium and far from maximal activation. The capacity of the nucleotide transporter is sufficient to transform the energy of the transmembrane potential into a $\Delta G'_{(ctt-mito)}$ of 4.6 kJ/mol ATP, even in hearts that are functionally stimulated.

In the event of an inactivation of the ADP/ATP carrier, the remaining intact carrier molecules are expected to perform an increasingly asymmetric nucleotide transport, owing to the failing difference of the cytosolic-mitochondrial phosphorylation potentials. Depending on the percentage of carrier inactivation, a new equilibrium will be attained at a lowered transmembrane difference in phosphorylation potentials. The theoretical end-state would be a condition in which the intact portion of the ADP/ATP carriers releases only ATP and takes up only ADP. Any further inactivation of carrier molecules must then lead to a continuous decrease of the cytosolic-mitochondrial difference of $\Delta G'_{ATP}$ with no possibility of reaching a new equilibrium.

Some of the immunized hearts showed a much lower value of $\Delta G'_{(ctt-mito)}$ than the controls; in two cases the mitochondrial phosphorylation potential even exceeded that of the cytosol. These results demonstrate an antibody-induced loss of nucleotide transport activity via the ADP/ATP carrier.

### Table 1. External Heart Work, Oxygen Consumption, and Lactate Release of Working Heart Preparations

<table>
<thead>
<tr>
<th></th>
<th>Cardiac work (mJ/g · min)</th>
<th>Oxygen consumption (µmol/g · min)</th>
<th>Lactate release (µmol/g · min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control ($n=5$)</td>
<td>637±102</td>
<td>11.9±4.9</td>
<td>0.67±0.21</td>
</tr>
<tr>
<td>Immunized animals ($n=10$)</td>
<td>642±200</td>
<td>11.5±3.3</td>
<td>0.90±0.44</td>
</tr>
<tr>
<td>Immunized animals ($n=6$) (nucleotide transport inhibition in vitro)</td>
<td>619±255</td>
<td>11.1±3.3</td>
<td>1.07±0.53</td>
</tr>
</tbody>
</table>

Values are mean±SD of control hearts and of hearts from guinea pigs immunized with isolated ADP/ATP carrier. Hearts were stimulated by 0.08 µM norepinephrine.
Cytosolic ATP and phosphocreatine concentrations showed the tendency to decrease, and cytosolic ADP concentration the tendency to increase. However, the differences in phosphorylation potentials between control hearts and immunized animals were much more impressive for the mitochondrial than for the cytosolic values. The probable reason for the less altered cytosolic phosphorylation potential of ATP is the submaximal stimulation to which the isolated perfused working hearts were subjected. Subcellular nucleotide concentrations and phosphorylation potentials are known to depend on the level of metabolic activation. Since in the present study the perfusion conditions were to be as close to the in vivo situation as possible, it was necessary to attain a steady state with heart work equal for control and immunized hearts. This is also a prerequisite for comparison of the function of the ADP/ATP carrier in both collectives. The requirement of reaching equal heart work in all hearts precluded stimulation to their individual maximum performance. Thus, only a shift in subcellular nucleotide relations rather than a true deficit of cytosolic high-energy phosphates was observed. At the time of study, none of the immunized animals had developed any overt signs of heart insufficiency.

There is no correlation between the titer of antibodies detected by radioimmunoassay and the antibodies' functional activity in vitro and in vivo. The lack of correlation can best be explained by the existence of several antibody populations displacing each other from their nearby binding sites according to their concentrations and affinities. The occurrence of such different populations of antibodies with nearly identical, overlapping binding sites but with different functional effects has already been demonstrated in patients with primary biliary cirrhosis. Correspondingly, we observed changes in the intracellular adenine nucleotide concentrations only in the isolated working hearts of those immunized guinea pigs whose sera inhibited the ADP/ATP carrier of isolated mitochondria. These shifts were not apparent in the hearts of those immunized guinea pigs with antibodies that showed

**Table 2. Subcellular Distribution of Myocardial High-Energy Phosphates in Isolated Perfused Guinea Pig Hearts**

<table>
<thead>
<tr>
<th></th>
<th>Nonimmunized controls</th>
<th>Immuneanimals</th>
<th>Immunized animals with inhibitory antibody production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cyt</td>
<td>Mit</td>
<td>Cyt</td>
</tr>
<tr>
<td>ATP</td>
<td>13.2±2.0</td>
<td>8.1±2.3</td>
<td>12.8±3.1</td>
</tr>
<tr>
<td>ADP</td>
<td>1.9±0.4</td>
<td>1.6±0.3</td>
<td>2.4±0.4</td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>61.8±7.9</td>
<td>5.1±0.5</td>
<td>55.1±11.2</td>
</tr>
<tr>
<td>ΔG' (cyt-mit)</td>
<td>4.6±0.5</td>
<td>2.1±2.1t</td>
<td>0.7±1.2t</td>
</tr>
</tbody>
</table>

Working heart preparations stimulated with 0.08 μM norepinephrine. Fractionation of tissue into cytosolic and mitochondrial fractions was accomplished by density gradient centrifugation in nonaqueous media. Data for cytosol (cyt) and mitochondria (mit) are given as concentrations (mM). Ratios of free cytosolic ATP/ADP were calculated from mass action ratio of creatine kinase reaction (see text). Phosphorylation potentials of ATP (ΔG') are given in kilojoule per mole ATP. Values are mean±SD, n=5 for controls, n=10 for all immunized animals tested, n=6 for those immunized animals showing an inhibition of ¹⁴C-ADP exchange in isolated mitochondria.

* p<0.05,
†p<0.005.

**Figure 6. Metabolite concentrations in cytosol and mitochondria obtained by nonaqueous fractionation.** Values for ATP, ADP, phosphate, phosphocreatine, and creatine are from isolated hearts of guinea pigs stimulated with norepinephrine (0.08 μM) in a working heart preparation. Symbols represent subcellular concentrations (mmol/l) of five control hearts (○) and of 10 hearts from guinea pigs immunized with ADP/ATP carrier with sera incapable of inhibiting (●) and sera capable of inhibiting (▲) ¹⁴C-ADP transport in isolated mitochondria.
no effect in the in vitro testing of the transmembrane nucleotide transport.

We conclude from the good coincidence between the experiments in vitro and in vivo that in six of 10 immunized guinea pigs antibodies against the ADP/ATP carrier entered into myocardial cells and caused a partial inhibition of carrier molecules. This assumption is confirmed by the results of direct immunofluorescence and immunoperoxidase staining, which showed depositions of immunoglobulins inside the cardiomyocytes. Electron microscopy of peroxidase-antiperoxidase–stained sections of myocardial cells from the immunized guinea pigs revealed that these immunoglobulins cling predominantly to mitochondrial membrane structures.

Some evidence for the intracellular occurrence of antibodies and for functional effects evoked by intracellular antibodies can be found in the literature.50–52 The intracellular uptake of antibodies may well occur by the mechanism of receptor-mediated endocytosis,53,54 This is known to be a mode of transport for many proteins, hormones, viruses, and toxins into different types of cells.39–57 In previous experiments we incubated isolated myocytes with gold-labeled antibodies against the ADP/ATP carrier.58 Electron microscopic sections

**FIGURE 7.** ATP/ADP ratios and phosphorylation potentials of ATP (in kiloJoules per mole ATP) in cytosol and mitochondria of hearts from control animals and from guinea pigs immunized with ADP/ATP carrier (hearts stimulated with norepinephrine 0.08 μM). Concentrations are calculated from subcellular metabolite contents as yielded by nonaqueous fractionation (see Figure 6). Symbols represent values of five control hearts (○) and of 10 hearts from guinea pigs immunized with ADP/ATP carrier with sera incapable of inhibiting (•) and sera capable of inhibiting (▲) 14C-ADP transport in isolated mitochondria.

**FIGURE 8.** Cytosolic and mitochondrial ATP concentrations (left panel) and ATP/ADP ratios (right panel) in hearts of guinea pigs immunized with ADP/ATP carrier (isolated perfused hearts stimulated with norepinephrine 0.08 μM). Values are mean±SD of five controls and six hearts of immunized guinea pigs showing inhibition of 14C-ADP transport activity in vitro.
Figure 9. Cytosolic-mitochondrial difference of phosphorylation potential of ATP (ΔG cyt-mito) in hearts of guinea pigs immunized with ADP/ATP carrier (isolated perfused hearts stimulated with norepinephrine 0.08 μM). Values are mean±SD of five controls and six hearts of immunized guinea pigs showing inhibition of 14C-ADP transport activity in vitro.

revealed the adherence of the gold particles to the cytoplasmic membrane and their appearance inside the cell. These latter particles are surrounded by membrane-like vesicular structures. As a prerequisite for an internalization of antibodies into myocardial cells, a binding site at the cell surface has to be postulated. Recent data of our group show that antibodies to the ADP/ATP carrier cross-react with a cell surface protein of 47 kDa molecular weight (Kühl et al, unpublished observations). This protein could be identified as the subunit of the connexon and might serve as the receptor for endocytosis of the immunoglobulins.

It is not certain whether the antibodies are taken up only by myocytes. However, an impairment of the function of other organs is not to be expected, even if the antibodies are taken up by other cells. This can be assumed from the organ specificity of the ADP/ATP carrier. Besides, new data seem to give evidence for an organ-specific autoantibodies to the ADP/ATP carrier exist in patients with dilated cardiomyopathy and myocarditis, as well as in patients with primary biliary cirrhosis,10,14,19,65 can now be extended by a functional aspect concerning the intact heart. Our data indicate that autoimmunity to the carrier protein may cause an imbalance between energy delivery and energy demand in cardiomyocytes and support the hypothesis that this kind of antibody-mediated disturbance of cellular functions is a crucial factor in the pathophysiology of myocarditis and dilated cardiomyopathy. The theoretical implications and clinical consequences based on this novel mechanism of an immunologically mediated dysfunction of the myocardial cell due to energy deficiency, without acute disturbance of its vital function, may be of great importance.

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**Key Words** • ADP/ATP carrier • nucleotide transport • energy metabolism • autoimmunity • myocarditis • cardiomyopathy
Antibodies to the ADP/ATP carrier, an autoantigen in myocarditis and dilated cardiomyopathy, penetrate into myocardial cells and disturb energy metabolism in vivo.

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