Coronary flow reserve and heart failure in experimental coxsackievirus myocarditis. A transthoracic Doppler echocardiography study

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VIRAL MYOCARDITIS IS USUALLY a mild disease, but sometimes it leads to irreversible myocardial injury, heart failure, and dilated cardiomyopathy (6). In addition to myocyte necrosis, structural damage of myocardial microcirculation, such as obstruction and luminal narrowing, has been observed in acute viral myocarditis in mice (4, 14, 20). Moreover, intracoronary guide wire measurements in patients with myocarditis and inflammatory cardiomyopathy have demonstrated that both inflammation and viral infection can cause endothelial dysfunction (12, 22). It has been hypothesized that dysfunction of coronary microcirculation resulting in focal ischemia-reperfusion contributes to myocardial injury in viral myocarditis (5, 15). However, the relationship between microvascular dysfunction and development of heart failure in myocarditis remains unknown.

Transthoracic Doppler echocardiography (TTDE) has been validated as a noninvasive tool to study coronary artery flow velocity and coronary flow reserve (CFR) in humans (2, 11, 17, 18). Reduced CFR, a ratio of coronary flow velocity during maximal vasodilatation with adenosine to baseline, indicates the presence of either a flow-limiting coronary artery stenosis or dysfunction of coronary microcirculation (10, 23). Recently, TTDE has been applied to study CFR in mice with atherosclerosis (7, 24).

To explore the role of dysfunction of coronary microcirculation in the course of viral myocarditis, we applied TTDE to measure CFR in mice and compared it with left ventricular systolic function in mice infected with coxsackievirus B3 (CVB3) variants causing either mild or severe forms of myocarditis.

**MATERIALS AND METHODS**

**Experimental model.** Thirty adolescent 5- to 7-wk-old male BALB/c mice from the Animal Center of the University of Turku were infected intraperitoneally with 2 × 10^4 plaque-forming units of two myocarditic variants of CVB3. We characterized the time courses of viral infection and myocarditis in this model in detail previously (1, 16). Ten mice were infected with the Nancy variant of CVB3 that causes mild myocarditis that is nonlethal. Twenty mice were infected with the Woodruff variant that causes severe myocarditis associated with high mortality. The local animal ethic committee approved the study, and the investigation conforms with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996).

**Study protocol.** All mice were studied by TTDE before infection. Five randomly chosen mice infected with either Nancy or Woodruff variants were studied again by TTDE 1 and 2 wk after infection. TTDE was performed with an Acuson Sequoia (Siemens Acuson, Mountain View, CA) and a 15-MHz linear transducer (15L8). Images were stored in a digital image archive (Kinet DX, Acuson Siemens) and analyzed later so that the observers were blinded to study groups and time points. All measurements were calculated as means of three cardiac cycles. Before TTDE, mice were fully anesthetized with ketamine (60 mg/kg) and diazepam (10 mg/kg), given intraperitoneally. The chest was mechanically shaved, mice were kept in the supine position, and normothermia was maintained with the use of a heating pad, a thermoregulating lamp, and a rectal thermometer. ECG leads were attached to extremities, and ECG was displayed in real time.

Adenosine (0.5 mg/ml; ITEM Development, Stocksund, Sweden) was infused intravenously via tail vein with an infusion pump at the rate of 0.32 mg·kg⁻¹·min⁻¹. Infusion was continued for a minimum of 2
min to ascertain that the maximum effect was achieved. A dose-response study comparing adenosine infusion rates (0.16, 0.32, and 0.64 mg·kg⁻¹·min⁻¹) was carried out in six noninfected mice to ascertain that the effective dosage was used. Mice were euthanized after TTDE using CO₂. The heart was excised, and transverse sections of the heart from the midventricular level were fixed in neutral buffered formaldehyde and embedded in paraffin for histological analysis.

M-mode echocardiography. Left ventricular dimensions and fractional shortening (FS) were measured in short-axis M-mode images of the left ventricle obtained at the level of mitral valve leaflet tips as previously described (8, 21). Coefficients of variation (CVs) for repeated measurements of FS by the same and by two independent analyzers were 0.07.

Doppler echocardiography. CFR was calculated as the ratio of peak diastolic flow velocity in the left coronary artery (LCA) during maximal vasodilatation induced by adenosine to resting flow velocity. Flow in the descending LCA was localized, and its course was followed under color Doppler mapping in the anterior wall of the left ventricle using modified long-axis views. The blood flow velocity spectrum in the middle LCA was then recorded by using pulsed-wave Doppler, both at rest and during adenosine infusion to measure diastolic flow velocities. Sample volume was kept as small as possible. Ultrasound beam and flow were aligned as parallel as possible. Angle correction was not used. CV for repeated measurements of CFR was successfully assessed in all animals before infection and again 1 or 2 wk after infection. Adenosine increased flow velocity in the middle LCA when compared with resting flow, as shown in Fig. 1 and Table 1. Before infection, average CFR was 2.4 (SD 0.6). The use of mean diastolic flow velocities instead of peak diastolic flow velocities resulted in comparable CFR values [2.4 (SD 0.4)]. In contrast, CFR was decreased 1 and 2 wk after infection with either virus variant at different time points. Correlations were calculated by using Spearman’s method. Values of *P* < 0.05 were considered statistically significant.

### RESULTS

Infection of mice with the Woodruff variant of CVB3 caused severe myocarditis characterized by large areas of necrosis and inflammation. Typically, 20–30% of all cardiomyocytes were affected. In contrast, infection with the Nancy variant resulted in mild myocarditis with few small necrotic foci (<1% of cardiomyocytes). The lesions were randomly located in all midventricular sections and across the ventricular wall. The results of histopathological grading are shown in Table 1. All mice infected with the Nancy variant survived, but eight (40%) mice infected with the Woodruff variant died during the follow-up. Heart rates did not differ significantly between baseline and after infection with either CVB3 variant (Table 1).

Cardiac function and left ventricular size. Representative images of M-mode echocardiography are shown in Fig. 1. As shown in Table 1 and Fig. 2, FS was decreased 1 and 2 wk after infection with either the Nancy (P < 0.05) or the Woodruff (P < 0.01) variant of CVB3 compared with baseline. FS did not differ between mice with mild and severe myocarditis before infection [0.51 (SD 0.03) and 0.54 (SD 0.02), respectively] or 1 wk after infection [0.44 (SD 0.02) and 0.43 (SD 0.03), respectively]. In contrast, 2 wk after infection, FS was significantly lower in severe than in mild myocarditis [0.31 (SD 0.03) vs. 0.47 (SD 0.02), P < 0.001]. Left ventricular diastolic diameter (LVDD) remained unchanged in mild myocarditis during the follow-up. When compared with the LVDD before infection, LVDD was increased 2 wk after infection in severe myocarditis [3.7 (SD 0.3) vs. 4.2 mm (SD 0.3), P < 0.05]. Thickness of interventricular septum or posterior wall of the left ventricle did not change after infection with either virus variant.

Assessment of CFR. CFR was successfully assessed in all animals before infection and again 1 or 2 wk after infection. Adenosine increased flow velocity in the middle LCA when compared with resting flow, as shown in Fig. 1 and Table 1. Before infection, average CFR was 2.4 (SD 0.6). The use of mean diastolic flow velocities instead of peak diastolic flow velocities resulted in comparable CFR values [2.4 (SD 0.4)]. In the dose-response study, mean CFR in six mice did not significantly differ when adenosine infusion rate varied from 0.16

### Table 1. Histopathological and echocardiographic characteristics of mice with mild and severe forms of myocarditis

<table>
<thead>
<tr>
<th></th>
<th>Mild Myocarditis</th>
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<th>Severe Myocarditis</th>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>1 wk</td>
<td>2 wk</td>
<td>Baseline</td>
</tr>
<tr>
<td><strong>Histopathological grade, [0–4 (median (range))]</strong></td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>415 (SD 59)</td>
<td>440 (SD 51)</td>
<td>430 (SD 49)</td>
<td>384 (SD 50)</td>
</tr>
<tr>
<td>Septal/posterior wall thickness, mm</td>
<td>0.5/0.5</td>
<td>0.5/0.5</td>
<td>0.5/0.5</td>
<td>0.5/0.5</td>
</tr>
<tr>
<td>LVDD, mm</td>
<td>3.7 (SD 0.3)</td>
<td>3.8 (SD 0.2)</td>
<td>3.7 (SD 0.2)</td>
<td>3.7 (SD 0.3)</td>
</tr>
<tr>
<td>LVSD, mm</td>
<td>1.8 (SD 0.2)</td>
<td>2.1 (SD 0.2)</td>
<td>2.0 (SD 0.2)</td>
<td>1.7 (SD 0.1)</td>
</tr>
<tr>
<td>FS</td>
<td>0.51 (SD 0.03)</td>
<td>0.44 (SD 0.02)</td>
<td>0.47 (SD 0.02)</td>
<td>0.54 (SD 0.02)</td>
</tr>
<tr>
<td>Resting LCA flow velocity, m/s</td>
<td>0.18 (SD 0.04)</td>
<td>0.17 (SD 0.05)</td>
<td>0.17 (SD 0.03)</td>
<td>0.18 (SD 0.05)</td>
</tr>
<tr>
<td>Hyperemic LCA flow velocity, m/s</td>
<td>0.43 (SD 0.11)</td>
<td>0.37 (SD 0.12)</td>
<td>0.49 (SD 0.05)</td>
<td>0.43 (SD 0.12)</td>
</tr>
<tr>
<td>CFR</td>
<td>2.5 (SD 0.3)</td>
<td>2.1 (SD 0.3)</td>
<td>2.4 (SD 0.6)</td>
<td>2.4 (SD 0.4)</td>
</tr>
</tbody>
</table>

Values are means (SD); *n*, number of mice. LVDD and LVSD, left ventricular diastolic and systolic diameter, respectively; FS, fractional shortening; LCA, left coronary artery; CFR, coronary flow reserve.
[2.3 (SD 0.6)] to 0.32 [2.5 (SD 0.4)], or to 0.64 mg·kg⁻¹·min⁻¹ [2.4 (SD 0.5)]. Heart rate remained similar at rest [416 beats/min (SD 75)] and during adenosine infusion rates of 0.16 [421 beats/min (SD 46)], 0.32 [433 beats/min (SD 49)], or 0.64 mg·kg⁻¹·min⁻¹ [419 beats/min (SD 50)]. There were no deaths during or immediately after adenosine infusion.

**CFR and myocarditis.** When compared with those before infection, resting flow velocities in the LCA did not differ after infection with either virus variant, as shown in Table 1. In contrast, adenosine-induced flow velocity was significantly lowered 1 wk after infection with either virus variant (P < 0.01). Thus CFR was reduced in all mice 1 wk after infection with either virus variant when compared with baseline, as shown in Table 1 and Fig. 2. At this time, average CFR had decreased from 2.5 (SD 0.3) to 2.1 (SD 0.3) (P < 0.05) after infection with the Nancy variant and from 2.4 (SD 0.4) to 1.4 (SD 0.1) (P < 0.01) after infection with the Woodruff variant. CFR remained low 2 wk after infection with the Woodruff variant [1.6 (SD 0.2), P < 0.05], whereas it was comparable to baseline in mice infected with the Nancy variant [2.4 (SD 0.6)].

When compared with that in mild myocarditis, CFR was significantly more reduced in severe myocarditis both 1 wk and 2 wk after infection (P < 0.01), as shown in Table 1 and Fig. 2. Thus reduced CFR 1 wk after infection preceded the progressive worsening of systolic function and the increase of LVDD in severe myocarditis. Two weeks after infection, reduced CFR significantly correlated with reduced FS (r = −0.65, P < 0.05). Reduced CFR also correlated with increased histopathological severity of myocarditis 1 wk (r = −0.80, P = 0.01) and 2 wk (r = −0.73, P < 0.05) after infection.

**DISCUSSION**

We found that CFR is reduced in experimental CVB3 myocarditis. To our knowledge, this is the first report to demonstrate that noninvasive TTDE with adenosine can detect dysfunction of coronary microcirculation in mice. Low CFR in the acute phase was associated with dilatation and progressive deterioration of systolic function of the left ventricle, indicating that dysfunction of coronary microcirculation is a determinant of poor outcome in viral myocarditis.

Infection of mice with CVB3 is the most common experimental model of myocarditis that has provided important insights into the pathogenesis of human disease (1, 6, 15, 16). Both viral infection of the myocardium and the associated inflammatory response contribute to the pathogenesis of disease. Previous studies (4, 14, 20) using microperfusion tech-
with severe myocarditis. Two weeks after infection, CFR was reduced only in mice with mild myocarditis (open bars), those with severe myocarditis (shaded bars) showed progressive worsening of fractional shortening together with an increase in LVDD 2 wk after infection (A and B). Infection of mice with either coxsackievirus variant resulted in a decrease in coronary flow reserve (CFR) 1 wk after infection (C). However, CFR was significantly more reduced in mice with severe myocarditis. Two weeks after infection, CFR was reduced only in mice with severe myocarditis. *P < 0.05; **P < 0.01.

Fig. 2. Mice infected with either virus variant had comparable reductions in fractional shortening 1 wk after infection (A). However, in contrast to mice with mild myocarditis, those with severe myocarditis (shaded bars) showed progressive worsening of fractional shortening together with an increase in LVDD 2 wk after infection (A and B). Infection of mice with either coxsackievirus variant resulted in a decrease in coronary flow reserve (CFR) 1 wk after infection (C). However, CFR was significantly more reduced in mice with severe myocarditis. Two weeks after infection, CFR was reduced only in mice with severe myocarditis. *P < 0.05; **P < 0.01.

Techniques have shown structural changes in myocardial microcirculation, such as obstruction and narrowing of small arteries, during acute viral myocarditis in mice. Intracoronary measurements have provided evidence of endothelial dysfunction in response to acetylcholine in epicardial coronary arteries of some patients with histologically proven myocarditis (12). More recently, intracoronary guide wire study demonstrated endothelial dysfunction in patients with inflammatory cardiomyopathy and persistent myocardial viral infection (22). However, the significance of microvascular damage in the development of cardiac dysfunction due to myocarditis remains unknown.

To study the effects of CVB3 infection on function of coronary microcirculation, we used TTDE to measure CFR after maximal vasodilatation induced by adenosine. This noninvasive approach has been extensively validated in various clinical conditions in humans (23) and, recently, in mice with atherosclerotic coronary arteries (7, 24). In the absence of significant stenosis in epicardial coronary arteries, reduced CFR reflects a dysfunction of coronary microcirculation (9, 23). Although adenosine may not be the most sensitive drug to detect microvascular dysfunction because its potent vasodilator effect on small arterioles may overwhelm vasoconstriction due to other causes (23), its advantage over acetylcholine is its suitability for intravenous use. Thus it is the drug of choice for noninvasive studies. We could visualize blood flow in the middle LCA in all mice by TTDE, and the flow velocity profile obtained with pulsed-wave Doppler was clearly distinct from mitral inflow and left ventricular outflow tract. CFR values obtained during adenosine-induced vasodilatation were highly reproducible. A previous study by Wikström and coworkers (24) found that a dose of 0.16 mg·kg\(^{-1}\)·min\(^{-1}\) was enough to cause maximal vasodilatation in mice. However, doses up to 0.32 mg·kg\(^{-1}\)·min\(^{-1}\) did not exert any systemic hemodynamic adverse effects. We used an adenosine dose of 0.32 mg·kg\(^{-1}\)·min\(^{-1}\), because in our dose-response study, it appeared to provide most consistent results in repeated measurements. This dose did not affect heart rate (≈400 beats/min) during ketamine-diazepam anesthesia, which is close to that reported in mice in the physiological state (8). Adenosine was well tolerated as none of the mice died during or immediately after infusion, allowing repeated assessment of CFR. CFR in noninfected BALB/c mice was in line with a previous study (24) using TTDE in healthy C57BL/6 mice. We found a consistent reduction of CFR in the acute phase of myocarditis after infection with either CVB3 variant. This was due to reduced flow velocity during adenosine infusion, whereas resting flow velocity remained unchanged. Thus TTDE can be used to study CFR and microvascular dysfunction caused by viral myocarditis in a noninvasive fashion.

To study the significance of coronary microvascular dysfunction in the development of cardiac injury and dysfunction caused by myocarditis, we compared CFR with histopathology and left ventricular function in mild and severe disease forms. Echocardiography plays a critical role in the assessment of myocarditis and its complications in the clinical practice (19). It has also become a routine tool to study various models of cardiac disease in the mouse (8, 21), but the experimental murine model of viral myocarditis remains uncharacterized. We found comparable reduction of systolic function in mild and severe forms of myocarditis 1 wk after infection, but only the severe myocarditis was associated with a progressive worsening of systolic function and dilatation of the left ventricle 2 wk after infection. CFR was significantly more reduced in severe than in mild myocarditis both 1 and 2 wk after infection, correlating closely with histopathological severity of myocardial injury. One week after infection, strongly reduced CFR preceded the development of progressive heart failure, indicating that early microvascular dysfunction may be an important determinant of poor outcome in viral myocarditis. Whether reduced CFR can be detected by TTDE in patients with severe myocarditis and cardiac dysfunction remains to be seen.

The observed microvascular dysfunction may be related to the replication of CVB3 in endothelial cells during acute myocarditis (13). Consistent with this, mice infected with the
Woodruff variant of CVB3 that had strongly reduced CFR also show more than 10 times higher titers of infectious virus in the myocardium than mice infected with the Nancy variant that had milder reductions of CFR (1, 16). However, CFR remained low in the severe disease form 2 wk after infection, despite the fact that virus was no longer detectable in the heart (1, 16). Thus there are likely other factors than the viral infection itself that contribute to microvascular dysfunction. These may be related to continuing myocardial inflammation (14) or damage to the microvasculature sustained in the acute phase (3, 12). Microvascular abnormalities have also been observed in cardiomyopathies of various etiologies, for example in Chagas’ disease (9). Interestingly, CVB3 is able to cause a long-lasting disease (9). Coxswievirus B3 infection compromises endothelial-dependent vasodilatation of coronary resistance arteries. J Med Virol 47: 251–259, 1995.


