EXPERIMENTAL COXSACKIE B VIRAL MYOCARDITIS IN CYMOMOLGUS MONKEYS

TATSUO HOSHINO, M.D., CHUICHI KAWAI, M.D.
AND MASAO TOKUDA, M.D.

In 11 of 13 cynomolgus monkeys inoculated with coxsackievirus B3 and/or B4, myocarditis was proved histologically. Myocarditis was evident the first 10 days after inoculation and left chronic sequelae; moderate myocardial cellular hypertrophy with an increase of connective tissue with focal distribution, and some residual inflammatory foci were found 5 months after viral inoculation. Virus was recovered from the heart on the 4th day but not on the 10th day, while serum neutralizing antibody rose significantly over 2 or 3 weeks in most of the inoculated monkeys.

Electrocardiographic abnormalities were found in all 11 monkeys in which the myocarditis was proved histologically. These abnormalities were usually transient and were detected most often in the ST-T segment of the right-side chest leads, corresponding to the anatomical distribution of the myocarditis.

Since the recognition of a relation of coxsackievirus group B to cardiac abnormalities in newborn infants in South Africa,1,2 many cases of myocarditis and pericarditis have been reported both in children and in adults with Coxsackie B viral infection. Coxsackie B viruses are now considered to be the most common cause of viral myocarditis in man.3

In experimental murine Coxsackie B viral myocarditis, long-term residual effects of the viral infection have already been detected,4,5 supporting the hypothesis that some cases of idiopathic cardiomyopathy in man may represent the late sequelae of viral infection.6,7

In this experiment we studied coxsackievirus B3 (CVB3) and/or B4 (CVB4) myocarditis using 13 cynomolgus monkeys to elucidate acute and long-term histological sequelae of myocarditis, and followed them up electrocardiographically.

MATERIALS AND METHODS

Viruses: CVB3 (T-253K/70 strain), used in this experiment, was isolated in newborn mice by one of us (M.T.) from the feces of a healthy 28-year-old female in Moulmain, Burma, in 1970.8 The virus was used in its 7th to 11th mouse passage. CVB4 (Kyoto/568K/70 strain) was isolated in HeLa cells by one of us (M.T.) from the feces of a 7-year-old boy suffering from vomiting in Kyoto, Japan, in 1970.9 The virus was used in its 13th mouse passage. The infectivity titer of CVB3 for HeLa cells was 10^7.0–7.5 TCID_{50}/0.1 ml and that of CVB4 10^6.5 TCID_{50}/0.1 ml.

Monkeys: Thirteen healthy cynomolgus monkeys imported from Malaysia were used.

Key Words:
Coxsackie B virus
Viral myocarditis
Electrocardiogram
Cynomolgus monkeys

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The Third Division, Department of Internal Medicine, Faculty of Medicine, and *The Institute for Virus Research, Kyoto University, Kyoto 606, Japan
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Present mailing address: Tatsuo Hoshino, M.D., Kansui Denryoku Hospital, 2-1-7 Fukushima, Fukushima-ku, Osaka 553, Japan

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### TABLE 1 SUBJECT PROFILE

<table>
<thead>
<tr>
<th>Monkey No.</th>
<th>Sex</th>
<th>Inoculated virus</th>
<th>Day of sacrifice or death</th>
<th>Body weight (g)</th>
<th>Heart weight (g)</th>
<th>Neutralizing antibody Control 2 or 3 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>m</td>
<td>CVB 3</td>
<td>4†</td>
<td>2,300</td>
<td>9.3</td>
<td>nd, nd</td>
</tr>
<tr>
<td>2</td>
<td>m</td>
<td>CVB 3</td>
<td>10</td>
<td>2,700</td>
<td>10.2</td>
<td>&lt;4, &lt;4</td>
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<tr>
<td>3</td>
<td>m</td>
<td>CVB 3</td>
<td>21</td>
<td>2,500</td>
<td>10.8</td>
<td>&lt;4, 64</td>
</tr>
<tr>
<td>4</td>
<td>m</td>
<td>CVB 3</td>
<td>28</td>
<td>3,000</td>
<td>10.6</td>
<td>nd, nd</td>
</tr>
<tr>
<td>5</td>
<td>f</td>
<td>CVB 3</td>
<td>35</td>
<td>2,600</td>
<td>9.3</td>
<td>&lt;4, 16</td>
</tr>
<tr>
<td>6</td>
<td>m</td>
<td>CVB 3</td>
<td>41</td>
<td>2,600</td>
<td>8.6</td>
<td>&lt;4, 512</td>
</tr>
<tr>
<td>7</td>
<td>m</td>
<td>CVB 3</td>
<td>60</td>
<td>2,300</td>
<td>8.1</td>
<td>&lt;4, 64</td>
</tr>
<tr>
<td>8</td>
<td>f</td>
<td>CVB 4</td>
<td>2†</td>
<td>1,900</td>
<td>—</td>
<td>nd, nd</td>
</tr>
<tr>
<td>9</td>
<td>m</td>
<td>CVB 4</td>
<td>15†</td>
<td>2,000</td>
<td>—</td>
<td>64, 256</td>
</tr>
<tr>
<td>10</td>
<td>f</td>
<td>CVB 4</td>
<td>20</td>
<td>2,200</td>
<td>10.6</td>
<td>&lt;4, 4</td>
</tr>
<tr>
<td>11</td>
<td>f</td>
<td>CVB 4</td>
<td>35</td>
<td>3,200</td>
<td>9.7</td>
<td>4, 8</td>
</tr>
<tr>
<td>12</td>
<td>f</td>
<td>1) CVB 3*</td>
<td></td>
<td>2,000</td>
<td>—</td>
<td>CVB3 &lt; 2, 64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) CVB 4</td>
<td>153</td>
<td></td>
<td></td>
<td>CVB4 nd, nd</td>
</tr>
<tr>
<td>13</td>
<td>m</td>
<td>1) CVB 4**</td>
<td></td>
<td>2,500</td>
<td>8.8</td>
<td>CVB4 16, 64</td>
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<td></td>
<td></td>
<td>2) CVB 3</td>
<td>35</td>
<td></td>
<td></td>
<td>CVB3 nd, nd</td>
</tr>
</tbody>
</table>

*CVB 3 had been inoculated intraperitoneally 43 days before CVB 4 inoculation.

**CVB 4 had been inoculated intravenously 36 days before CVB 3 inoculation.

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After virus inoculation, they were caged individually in a room at a temperature maintained between 22° and 24°C.

Experimental Infections: Monkeys were inoculated with CVB3 and/or CVB4 via the posterior tibial vein (Table I). Each monkey received 1.0 ml of the undiluted stock virus.

Virus Re-isolation from the Heart and Serologic Tests: Heart tissue was ground with sea sand in Eagle’s medium and the medium was adjusted to obtain a 20% tissue suspension (wt/vol). The suspension was centrifuged at 2,000 rpm for 10 min and subsequently at 7,000 rpm for 30 min at 4°C. A 0.3 ml amount of the supernatant was injected into the HeLa cell tube cultures which were observed daily for the cytopathic effect of the virus for 7 days. Blood samples were obtained serially from the femoral artery of each monkey for the neutralization tests with CVB3 and CVB4.

Histological Examination: The hearts, obtained at necropsy or after sacrifice by exsanguination on the days indicated in Table I, were fixed in a 10% formalin solution and were cut coronally parallel to the base, producing 5 blocks from each heart. After embedding in paraffin, 20 serial sections of 4 micron-thickness were made from the upper surface of each block. Sections were stained with hematoxylin and eosin (HE).

Electrocardiogram (ECG): A standard 12-lead ECG was recorded at least once a week by placing needle electrodes subcutaneously while the monkey was fixed supinely on a board using a Siemens Mino graf Minor 3 (3 channel jet ink writer) at a paper speed of 50 mm/sec. In the first 4 monkeys studied the ECG was taken under cyclohexylamine anesthesia, 10 mg/kg intramuscularly. In the other monkeys the ECG was recorded without anesthesia because of an improved recording technique.

Echocardiogram: M-mode echocardiogram was obtained repeatedly from 2 monkeys in the conscious state on a Polaroid camera using an Aloka SSD-110 Echocardiographer and an infantile transducer of 3.5 MHz. The transducer was placed in the 3rd or 4th intercostal space near the left sternal border, with the monkey in the 30° to 90° left anterior oblique position.

RESULTS

**Histological Findings**

Myocarditis was proved histologically in 11 of
the 13 inoculated monkeys. Although myocarditis was not seen in one monkey which died suddenly 4 days after CVB3 inoculation (No. 1), the virus was recovered from the heart of this monkey. On the other hand, myocarditis was obvious in another monkey which showed ST elevation in the right-side chest leads on the 9th day after CVB3 inoculation and which was sacrificed on the following day (No. 2), although the virus could no longer be recovered from the heart. In this monkey, histological examination showed small patchy lesions located predominantly in the epicardial as well as in the subendocardial regions of the right ventricle.

Myocarditis was prominent in another monkey which exhibited marked sinus bradycardia on the 14th day after CVB4 inoculation and died on the next day (No. 9) (Fig. 1). Myocarditis was also found in the monkeys sacrificed between 20 and 60 days after viral inoculation (Table II).

Cell infiltration in the myocardium appeared milder in another monkey sacrificed 60 days after the inoculation (No. 7) than that in the monkeys which died or were sacrificed earlier.

Moderate hypertrophy of the myocardial cell, an increase of interstitial connective tissue
TABLE II  ELECTROCARDIOGRAPHIC ABNORMALITIES AND DISTRIBUTION OF THE INFILAMMATORY LESIONS IN THE HEART

<table>
<thead>
<tr>
<th>Monkey No.</th>
<th>ECG abnormalities</th>
<th>Inflammatory lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Atrium</td>
</tr>
<tr>
<td>1</td>
<td>nd</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>ST elevation (V1–3)</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Sinus tachycardia</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>T inversion (V2) and decreased T amplitude (II, aVR, V3)</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Decreased T amplitude (V1, 2)</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Decreased T amplitude (V1–4)</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>Decreased T amplitude (II, V1–6)</td>
<td>++</td>
</tr>
<tr>
<td>8</td>
<td>Atrial premature contraction</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Sinus bradycardia</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>T inversion (V1)</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>Decreased T amplitude (I, II, aVR, V1–5)</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>T inversion (V2–6)</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>Decreased T amplitude (V1–4)</td>
<td>0</td>
</tr>
</tbody>
</table>

0 = no inflammatory changes; ++ = few small focal inflammatory lesions; +++ = several small inflammatory lesions; ++++ = large inflammatory lesions. LV = left ventricle; IVS = interventricular septum; RV = right ventricle; SV = semilunar valve; Ao = aorta; PA = pulmonary artery; RCA = right coronary artery; nd = not examined repeatedly.

with focal distribution and some residual inflammatory foci were found in another monkey which showed transient T wave inversion on the chest leads ECG and which was sacrificed 153 days after CVB4 inoculation (No. 12) (Fig. 2). Although CVB3 had been injected intraperitoneally 43 days before CVB4 inoculation in this monkey, the electrocardiographic abnormalities were not manifest until CVB4 inoculation.

Neutralizing Antibody

Seven of 10 monkeys examined showed a significant rise in serum homotypic neutralizing antibody 2 or 3 weeks after viral inoculation (Table I). Two other monkeys showed a tendency towards a rise in antibody titer.

Distribution of the Inflammatory Lesions in the Heart (Table II)

The myocardial lesions were usually small and patchy with the main lesions located usually around the right ventricular cavity, particularly in the subendocardium. Major lesions of the left ventricle were also located in the subendocardium. Small inflammatory foci were scattered in the epicardial as well as in the subendocardial regions but inflammation located in the middle layer of the ventricle was rare.

The inflammatory lesions were occasionally found in the atrium, the atrial ganglion cells (Fig. 3), the coronary artery (Fig. 4), the pulmonary artery and the endocardium. However, we could not find any valvular fusion or coronary arterial obstruction due to inflammation.

Difference between Cardiac Lesions Caused by CVB3 and Those by CVB4

Cardiac lesions caused by CVB3 usually appeared as well circumscribed necrotic myocarditis. On the other hand, CVB4 induced inter-
Fig.2. (a): Serial ECGs in monkey No.12. T wave inversion was recorded in leads V2–6 one week after CVB4 inoculation, which recovered 2 weeks later.

(b): The left ventricular myocardium 153 days after CVB4 inoculation. The right upper and left lower parts of this figure show moderate hypertrophy of the myocardial cells and increased interstitial connective tissue.

HE x120.

stially infiltrating myocarditis, commonly showing lymphocytes and mononuclears as the infiltrating cells. A small number of plasma cells were scattered in old myocarditic lesions.

**ECG and Echocardiogram**

In all 11 monkeys examined electrocardiographic abnormalities appeared after infection, and in all of them myocarditis was proved histologically. Changes in ST segment or T waves were observed most frequently; sinus bradycardia (Fig. 1), sinus tachycardia and atrial premature contraction (Fig. 3) were detected in one case each (Table II). The ST-T changes were usually
detected on the right-side chest leads, corresponding to the anatomical distribution of the myocarditis. These electrocardiographic abnormalities were recorded 4 to 21 days after viral inoculation and persisted for 4 to 21 days.

Cyclohexylamine anesthesia alone did not cause any significant electrocardiographic alterations.

M-mode echocardiography performed serially on 2 of the CVB3-inoculated monkeys (Nos. 2 and 7) detected no abnormality in the mitral valvular and left ventricular wall motions.

DISCUSSION

Viral myocarditis has been related to the pathogenesis of idiopathic cardiomyopathy.\textsuperscript{3,6,7} In experimental viral myocarditis, Wilson et al.\textsuperscript{4} demonstrated persistent inflammation progressing to myocardial fibrosis for at least 6 months after inoculation in murine infection with CVB3. Furthermore, Tokuda et al.\textsuperscript{5} demonstrated persistence of dystrophic mineralization and myocardial fibrosis with fiber disarray for more than 2 years in murine Coxsackie B viral myocarditis. However, few studies of experimental viral myocarditis followed by examining ECG have been reported previously.\textsuperscript{10,11} To study acute viral myocarditis and its chronic sequelae in monkeys histologically as well as electrocardiographically, we inoculated CVB3 and/or CVB4 into cynomolgus monkeys known to be susceptible to these viruses.\textsuperscript{10–13}

Detailed virological and histological studies of acute CVB4 viral myocarditis in cynomolgus monkeys have been reported by Lou et al.\textsuperscript{10} We obtained similar results in the acute stage, although prominent lesions were found more often around the right ventricular cavity, corresponding to the electrocardiographic abnormalities preponderant on the right-side chest leads; neither mineralized lesions in the myocardium nor lesions involving the all layers were seen.

We found myocarditic lesions most often in the subendocardial myocardium. The location of the lesions in the present study corresponded
well to that observed by Burch et al\textsuperscript{14} in infants and children with Coxsackie virus B myocarditis, i.e., the inner third of the myocardium was involved more often than the outer two thirds. As suggested by Levine\textsuperscript{15} relative hypoxia of the normal subendocardium or a heavier distribution of the virus in the blood than in the more remote myocardium may favor specific localization of viruses in that region.

In the chronic stage of myocarditis we have demonstrated persistent sequelae of focally distributed myocardial cell hypertrophy and increased interstitial connective tissue 5 months after viral inoculation, when the electrocardiographic signs of myocardial injury had already disappeared.

Virus recovery from the heart was possible only for a short time after the inoculation, confirming the experiments with cynomolgus monkeys by Lou et al\textsuperscript{10} and with mice by Wilson et al\textsuperscript{13}, while the inflammatory lesions of the myocarditis persisted long after disappearance of the virus. Cellular immunity may have played an important role in the pathogenesis of Coxsackie B viral myocarditis in the cynomolgus monkeys as demonstrated in murine experiments\textsuperscript{16–21} and in the baboon\textsuperscript{22} though humoral factors should not be neglected in its pathogenesis\textsuperscript{6}. Abnormal electrocardiographic changes were not detected by Lou et al\textsuperscript{10} in cynomolgus monkeys with histologically proven experimental CVB4 viral myocarditis on the ECG recorded every 2 weeks. However, we did find abnormalities in cynomolgus monkeys with CVB3 and/or CVB4 viral myocarditis by recording standard 12-lead ECG at least once a week. Since electrocardiographic abnormalities were usually mild and transient, frequent repetition of reliable recordings seemed essential for their detection. They were nonspecific for viral myocarditis in monkeys as well as in humans\textsuperscript{3} occurring most often in the ST segment or T waves. In human myocarditis due to viral and Mycoplasma pneumoniae infections, Lewes et al\textsuperscript{23} described ECG signs which persisted for 2 to 14 days and occasionally for many months. The transiency and non-specificity of the abnormalities in both experimental and clinical viral myocarditis suggest that human viral myocarditis may pass undetected unless special attention is paid to its occurrence in viral infections.

At present serial electrocardiographic examinations seem to be the most sensitive method, though nonspecific, for the detection and follow up of viral myocarditis both in experimental animals and in humans.
Acknowledgments

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