Coxsackievirus B1-induced murine myositis: no evidence for viral persistence

Jan Zoll,1* Peter Jongen,2 Jochem Galama,1 Frank van Kuppeveld1 and Willem Melchers1

Departments of 1Medical Microbiology and 2Neurology, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

The persistence of coxsackievirus B1 in the muscles of mice with coxsackievirus B1-induced chronic myositis was investigated. Neonatal CD1 Swiss mice were inoculated with a myositis-causing variant of coxsackievirus B1 (Tucson strain). Hamstring muscle samples of diseased mice obtained at various times after inoculation were examined for the presence of infectious virus, viral RNA and histological abnormalities. Viral RNA was detected up to 4 weeks after initiation of infection, whereas virus could be isolated from hamstring muscles for up to 2 weeks. Thereafter no sign of infection was demonstrated although histological abnormalities remained present for the entire observation period of 16 weeks. That viral RNA was detectable for only 2 weeks after tissues became negative for infectious virus suggests that the infection slowly waned rather than the viral RNA persisting. Hence, it is concluded that coxsackievirus B1 plays an essential role in the initiation of myositis but not in the maintenance of the chronic phase.

Introduction

Enteroviruses are thought to be involved in the development of chronic diseases such as insulin-dependent diabetes mellitus, dilated cardiomyopathy, polymyositis/dermatomyositis and post-viral fatigue syndrome. It has been suggested that these diseases are caused by an autoimmune reaction possibly triggered by a transient enteroviral infection (Dalakas, 1991; Woodruff, 1980). However, during the last decade, several papers have described the detection of enteroviral RNA in muscle tissues of patients suffering from dilated cardiomyopathy (Jin et al., 1990; Kandolf et al., 1987), polymyositis/dermatomyositis (Bowles et al., 1987), and post-viral fatigue syndrome (Youssef et al., 1988), after the virus could no longer be isolated. They suggest that persistence of viral RNA might be involved in the pathogenesis of these diseases.

Several animal models have been developed, in which pancreatitis (Vella et al., 1992), cardiomyopathy (Gauntt et al., 1979) and myositis (Ray et al., 1979) are induced by infection with specific strains of coxsackie B viruses. Ray et al. (1979) described a murine model for myositis induced by a coxsackievirus B1 infection of suckling mice. Studies with that model revealed evidence that immunological reactions cause the chronic myositis (Strongwater et al., 1984; Ytterberg, 1987). However, in recent studies using the same murine model it was found that although viral RNA was still present in affected muscle fibres no infectious virus could be isolated from these samples (Tam et al., 1991). From this observation it was concluded that coxsackievirus B1 persisted after the acute phase of infection.

Recently, however, Koide et al. (1992) investigated the role of viral persistence during the development of murine cardiomyopathy. In this study the viral RNA could not be detected at more than 4 weeks after inoculation.

To investigate whether viral persistence indeed plays a pathogenic role in coxsackievirus B1-induced myositis, we examined muscle samples from infected mice for the presence of infectious virus, viral RNA and histological abnormalities.

Methods

**Virus strain.** A myotropic coxsackievirus B1 strain (Tucson strain) causing myositis in CD1 Swiss mice was kindly provided by L. Minnich and G. Ray, University of Arizona, Tucson, Arizona, U.S.A. (Ray et al., 1979). The virus was propagated in buffalo green monkey kidney (BGM) cells and subsequently stored in aliquots at −80 °C.

**Animal model.** Pregnant CD1 Swiss mice that were specific pathogen-free and random-bred were purchased from Charles River Wiga. Neonates were randomly chosen from different litters and inoculated intraperitoneally within 24 h after birth with 1000 p.f.u. coxsackievirus B1 in 0.1 ml PBS (P. Jongen, unpublished results).

Infected mice were randomly divided among the mothers in groups of 10 to 14 animals. At 2 weeks post-inoculation (p.i.), infected mice were examined for clinical signs of myositis i.e. abnormal posture and a waddling gait. Animals appearing healthy at 2 weeks p.i. were excluded from further experiments. At 1, 2, 4, 8 and 16 weeks p.i., infected mice were sacrificed for histological and virological examin-
Table 1. Semi-quantitative score for histological abnormalities in transverse sections of hamstring muscles

<table>
<thead>
<tr>
<th>Score</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infiltrating cells</td>
<td>0</td>
<td>&lt; 10</td>
<td>10-50</td>
<td>50-100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Necrotic muscle fibres</td>
<td>0</td>
<td>1-5</td>
<td>5-15</td>
<td>15-25</td>
<td>&gt; 25</td>
</tr>
<tr>
<td>Central nuclei</td>
<td>0</td>
<td>&lt; 25%</td>
<td>25-50%</td>
<td>50-75%</td>
<td>&gt; 75%</td>
</tr>
<tr>
<td>Atrophic muscle fibres</td>
<td>0</td>
<td>&lt; 25%</td>
<td>25-50%</td>
<td>50-75%</td>
<td>&gt; 75-100%</td>
</tr>
</tbody>
</table>

Table 2. Histological and virological examination of hamstring muscles from infected mice

<table>
<thead>
<tr>
<th>Time after infection (weeks p.i.)</th>
<th>Mouse no.</th>
<th>PCR</th>
<th>Virus titre (TCID&lt;sub&gt;50&lt;/sub&gt;)</th>
<th>Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Infiltrating cells</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>+</td>
<td>10&lt;sup&gt;6.2&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+</td>
<td>10&lt;sup&gt;6.0&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>+</td>
<td>10&lt;sup&gt;6.0&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>+</td>
<td>10&lt;sup&gt;6.0&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+</td>
<td>10&lt;sup&gt;6.0&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>+</td>
<td>10&lt;sup&gt;7.0&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>+</td>
<td>10&lt;sup&gt;7.0&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>+</td>
<td>10&lt;sup&gt;7.0&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+</td>
<td>10&lt;sup&gt;7.0&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>+</td>
<td>10&lt;sup&gt;8.0&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+</td>
<td>10&lt;sup&gt;8.0&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>+</td>
<td>10&lt;sup&gt;8.0&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>+</td>
<td>10&lt;sup&gt;8.0&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+</td>
<td>10&lt;sup&gt;8.0&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>+</td>
<td>10&lt;sup&gt;8.0&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+</td>
<td>10&lt;sup&gt;8.0&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>+</td>
<td>10&lt;sup&gt;8.0&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>+</td>
<td>10&lt;sup&gt;8.0&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+</td>
<td>10&lt;sup&gt;8.0&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>16</td>
<td>1</td>
<td>+</td>
<td>10&lt;sup&gt;8.0&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+</td>
<td>10&lt;sup&gt;8.0&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>+</td>
<td>10&lt;sup&gt;8.0&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>+</td>
<td>10&lt;sup&gt;8.0&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+</td>
<td>10&lt;sup&gt;8.0&lt;/sup&gt;</td>
<td>3</td>
</tr>
</tbody>
</table>

Gluteus, hamstring and quadriceps muscles from both hindlimbs were removed and divided into three equal parts each. For histological examination and in situ hybridization one part was placed in buffered formalin, paraffin-embedded and sectioned at 6 μm. For PCR analysis examination one part was immediately frozen into liquid nitrogen and stored at −80 °C until use. Another part was placed into 1 ml of Hanks' medium supplemented with 0.1% yeast extract, 0.5% lactalbumin hydrolysate and 0.5% gelatin and stored at −80 °C until use for virus isolation.

Histological examination. From every muscle sample transverse sections were stained with haematoxylin-phloxin-saffron and examined using a light microscope (magnification ×250). Sections were read under code. Degrees of mononuclear cell infiltration, necrosis, central nuclei and muscle fibre atrophy were scored using a five-grade scale (Table 1).

In situ hybridization. Deparaffinized muscle sections were hybridized as described (Kandolf et al., 1987). Briefly, before hybridization the slides were treated with 0.2 M HCl for 20 min, 2×SSC at 70 °C for 30 min, 20 mM-Tris-HCl pH 7.4, 2 mM CaCl<sub>2</sub> and 1 μg/ml proteinase K at 37 °C for 15 min. Slides were washed in distilled water and dehydrated.

An infectious coxsackievirus B1 cDNA clone (kindly provided by Dr A. Nomoto, University of Tokyo, Japan) (Iizuka et al., 1991) was digested with Sal<sub>1</sub>. A 7.5 kb fragment representing the entire coxsackievirus B1 genome was purified by agarose gel electrophoresis and subsequently labelled with [α-35S]dCTP by nick-translation (Sambrook et al., 1989). Radiolabelled DNA fragments of 100 to 400 bp were generated. Hybridization was performed using a mixture of DNA and 0.1×SSC.
Coxsackievirus-induced myositis

Fig. 1. Hamstring muscle. Haematoxylin-phloxin staining. (a) Massive infiltration of mononuclear cells (centre), muscle fibre atrophy, necrotic fibres and loss of fibre 2 weeks p.i. Bar represents 100 μm. (b) Numerous mononuclear cells surrounding and partially invading necrotic muscle fibres 4 weeks p.i. Bar represents 50 μm. (c) Endomysial infiltrate of mononuclear cells 8 weeks p.i. Bar represents 50 μm. (d) Muscle fibres with internal nuclei, fat cell replacement and sporadic myophagia, reflecting ongoing myopathy 16 weeks p.i. Bar represents 100 μm.

containing 200 ng/ml denatured 35S-labelled DNA probe in 10 mM-Tris–HCl pH 7.4, 50% (v/v) formamide, 0.6 mM-NaCl, 1 mM-EDTA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.05% BSA, 10% dextran sulphate, 10 mM-DTT, 200 μg/ml salmon sperm DNA and 100 μg/ml rabbit liver tRNA. After incubation at 25 °C for 36 h slides were washed at 30 °C for 18 h in 10 mM-Tris–HCl pH 7.4, 2 x SSC, 50% formamide and 1 mM-EDTA followed by 2 x SSC at 55 °C for 1 h. Hybridized muscle preparations were autoradiographed as described (Kandolf et al., 1987) and Giemsa-stained.

PCR. RNA was extracted from muscle samples using a single extraction with guanidinium thiocyanate–phenol–chloroform according to the method of Chomczynski & Sacchi (1987). Reverse transcriptase reactions and PCR were performed according to Zoll et al. (1992). Briefly, cDNA was synthesized in a 20 μl reaction mixture containing 75 mM-KCl, 50 mM-Tris–HCl pH 8.3, 3 mM-MgCl₂, 10 mM-DTT, 0.2 mM (each) deoxynucleoside triphosphate (Boehringer Mannheim), 50 pmol of antisense oligonucleotide primer (5' ATTGTCACCATAAGCACCCA 3'), 50 pmol of antisense oligonucleotide primer (5' AAAAAACGAACCAATGAGTGA 3'), 5 units of avian myeloblastosis virus reverse transcriptase (Promega) and extracted RNA. After incubation at 37 °C for 60 min, 80 μl of the PCR mixture was added. The PCR mixture contained 50 mM-KCl, 10 mM-Tris–HCl pH 8.9, 3.6 mM-MgCl₂, 0.2 mM (each) deoxynucleoside triphosphate, 100 μg of BSA/ml, 80 pmol of sense oligonucleotide primer (5' TCTTCCGGCCCTGAACTGCG 3'), 40 pmol of antisense oligonucleotide primer and 0.2 units of SuperTaq DNA polymerase (HT Biotechnology). RNA–cDNA hybrids were denatured at 94 °C for 5 min. The amplification was performed in 40 cycles consisting of denaturation for 1 min at 94 °C, primer annealing for 1 min at 42 °C and elongation for 2 min at 72 °C. The reactions were analysed by electrophoresis in 2% agarose gels and Southern blot hybridization. A 32P-end-labelled oligonucleotide (5' AAACACCGGAACCAAAAGTA 3') was used as the internal probe (Zoll et al., 1992).

Virus isolation. Muscle samples were homogenized using sterile sand, suspended in 1 ml of Earle’s medium and titrated in 10-fold dilutions on BGM monolayers in triplicate in a volume of 100 μl (Schmidt, 1979). TCID₅₀ values were calculated according to Reed & Muench (1938).

Results

Animal model

Neonatal CD1 Swiss mice from 10 litters were intraperitoneally inoculated with 1000 p.f.u. coxsackievirus B1 (Tucson strain) in 0–1 ml PBS. Within the first 2 weeks mortality was 40%, and morbidity, expressed as the percentage of surviving animals demonstrating abnormal
The number of mononuclear infiltrating cells, necrotic fibres and atrophic fibres reached a maximum within the first 2 weeks p.i. At least until 16 weeks p.i. infiltrating cells, necrosis and atrophy can be seen. The maximum number of fibres with central nuclei is reached at 8 weeks p.i. Mice inoculated with PBS developed no histological abnormalities.

Detection of virus and viral RNA
Muscle sections obtained 1 week p.i. were hybridized with a 35S-labelled DNA probe. The viral infection displays a focal character as shown in Fig. 2. Viral RNA is present in single muscle fibres whereas neighbouring fibres are not affected. Hamstring muscles were examined by virus isolation and PCR (Table 2).

One week p.i. all hamstrings tested contained viable virus and viral RNA. Two weeks p.i. viral RNA was detected in all of five hamstring muscles tested. Three of these five samples also contained viable virus as shown by virus isolation. The mean log10 virus titre in these three muscles, however, was decreased from 5-6 to 1-2.

Four weeks after inoculation all of 10 mice tested contained viral RNA in the hamstring muscles of one or both hindlegs whereas viable virus could no longer be isolated. Viral RNA was not detected in muscle more than 4 weeks p.i. Fig. 3 shows the hybridization results of PCR products for some muscle samples obtained at 4 and 8 weeks p.i.

Discussion
Polymyositis is an inflammatory disease of the skeletal muscle. It is the most frequently acquired muscular disease in adults and has a chronic progressive character.

Histological examination
Preliminary examination (P. Jongen, unpublished results) revealed that histological abnormalities were most extensive in the hamstring muscles. Therefore, hamstring muscles were used for further investigations. Table 2 gives the histological score and representative photographs are shown in Fig. 1(a to d).

Fig. 2. In situ detection of coxsackievirus B1 RNA in sections from the hamstring muscles 1 week p.i.

Fig. 3. Southern blot hybridization of coxsackievirus B1-specific products of RNA isolated from right hamstring muscle samples. (a) Four weeks p.i.; (b) 8 weeks p.i. Numbers indicate the different mice. Lanes indicated by +, muscle sample obtained from an infected mouse 1 week p.i.; lanes indicated by -, muscle sample from an uninfected mouse; lanes D, coxsackievirus B1 cDNA clone; lanes R, coxsackievirus B1 (Tucson) RNA. Size markers are indicated on the left.

gait and posture, was 60%. Animals appearing healthy at 2 weeks p.i. were excluded from further experiments. As a control, mice from two different litters were inoculated with 0.1 ml PBS only. At each time point after inoculation five to 10 mice were sacrificed. Skeletal muscles from both hindlegs were removed for further histological and virological examination.

Histological findings clearly showed a myositis continuing for at least 16 weeks that resembled poly-
myositis in man (Table 1, Fig. 1). In situ hybridization on affected hamstring muscle showed a focal distribution of the viral RNA, which is also described in human polymyositis (Bowles et al., 1987), and which is also found in viral murine myocarditis (Kandolf et al., 1987; Klingel et al., 1992). Infectious virus was isolated from the affected muscles until 14 days p.i. Beyond this time the virus can no longer be isolated, although viral RNA could still be detected by PCR up to 4 weeks p.i. These results are in agreement with Tam et al. (1991) who were also able to detect viral RNA up to about 31 days p.i. in this animal model. However, although histologically an ongoing myositis was found, viral RNA was no longer present beyond 4 weeks p.i. Thus, it appears that the maintenance of disease in this model is not associated with the persistence of entroviral RNA. Until now most studies on persistence of picornaviruses in murine models revealed similar results. Using an encephalomyocarditis virus variant, Cronin et al. (1988) induced myositis in mice. Virus was isolated at high titres 1 week p.i., but only at low titre 2 weeks p.i. Viral RNA was detected in skeletal muscle up to 3 weeks and only sporadically as late as 4 weeks p.i. Koide et al. (1992) detected viral RNA in a murine model of coxsackievirus B3-induced myocarditis, up to day 28 p.i. They described a decreasing number of positive animals with time: at day 14 p.i. five of six animals, and at day 28 p.i. only two of six animals were found positive for viral RNA. Thereafter, the viral RNA could no longer be detected in myocardial cells. A similar observation was made by Klingel et al. (1992). They described an ongoing myocarditis in the same animal model. Although the virus could not be isolated later than 15 days p.i., the viral RNA was still detectable up to 30 days p.i. However, in the acute phase of disease 13% of the myocardial cells were found to be infected, whereas in the chronic phase at day 30 p.i., only 0.01% of the myocardial cells were found positive for entroviral RNA.

In a previous study the sensitivity of the PCR was determined to be 100 genomic RNA molecules (Zoll et al., 1992). Although the presence of smaller amounts cannot be detected it seems unlikely that only a few RNA molecules persist in a limited amount of cells, in contrast with the more extensive necrosis observed after 4 weeks p.i. Thus, in the mouse models of myositis and myocarditis, the viral RNA can be detected for approximately 4 weeks p.i. Since virus can be isolated for 2 weeks p.i., it means that the virus "persists" for only 2 additional weeks.

Enteroviruses cause a lytic infection. The lytic infection is controlled by the immune system which will become active within 5 to 7 days. For reasons which are not fully understood, the lytic infection can be limited to a chronic productive one with focal necrosis and expression of viral antigens in both surviving and necrotic cells (Vella et al., 1992). Therefore, it may be that the virus can be trapped in a few cells which do not immediately lyse, probably due to defective virus replication. Several reports have described an abnormal production of equal amounts of positive and negative strands of entroviral RNA in patients with the post-viral fatigue syndrome as well as in coxsackievirus B3-induced myocarditis in the mouse (Cunningham et al., 1990; Klingel et al., 1992). The presence of a 1 : 1 molar ratio of the plus- and minus-strand RNA suggests the remaining presence of highly stabilized dsRNA intermediates which resist RNA degradation. The RNA will eventually be eliminated. Presumably this is what occurs in the murine after approximately 4 weeks. This indicates that the infection is waning.

Ytterberg and coworkers studied the murine myositis model extensively, especially the role of T cell activity (Ytterberg et al., 1987, 1988). These studies revealed that active virus and T cell activity were essential for the development of myositis. Mice with T cell deficiency, such as nude and athymic mice, did not develop myositis. This suggests that a chronic myositis may be caused by an immunologically mediated mechanism. In fact, a striking feature of all different animal models is the necessity for infectious virus, and in most cases T cell activity, to induce a chronic inflammation. The inflammation is demonstrated by histological parameters such as mononuclear infiltrating cells, necrosis, atrophy, etc. Histological abnormalities are visible in affected muscles for several months. However in this model, numbers of mononuclear infiltrates decrease with time indicating that the duration of the virus-induced chronic myositis is limited. Non-progressive gait abnormality observed several months after infection may be caused by the inflammation and corresponding loss of muscle tissue during the first 2 weeks of the infection. The presence of mononuclear infiltrating cells at 16 weeks p.i. indicates that the myositis is still occurring at this time.

Post-infectious autoimmunity has been postulated by many investigators and remains an important issue to be studied (Craighead et al., 1990). From studies in the murine cardiomyositis model it became clear that anti-heart antibody is generated in the myocarditic mouse (Paque & Miller, 1991), and that autoreactive cytotoxic T lymphocytes are generated, which can cause cardiac damage after adoptive transfer to uninfected mice (Loudon et al., 1991). In addition, several epitopes have been described that show cross-reactivity between coxsackie B viruses and cardiac myositis (Paque & Miller, 1991). The strain of coxsackie B virus used, as well as genetic factors of the host, are found to be of crucial importance. What makes a strain myocarditic or not is still poorly understood. The difference might reside in the
type of immune response mounted against the various strains (Chow et al., 1991). It is exciting to speculate that molecular characteristics of the virus are involved in the immunopathology.

Although the viral infection appears to be essential for the initiation of inflammation (Ytterberg, 1987), a permanent presence of the viral RNA does not contribute to the maintenance of the disease.

We are indebted to Professor Dr R. Kandolf for his advice in the in situ hybridization technique. This study was supported by the Prinzes Beatrix Fonds, The Netherlands (grants 89-2999 and 90-3137).

References


(Received 11 March 1993; Accepted 11 June 1993)