Myocarditis in newborn wild-type BALB/c mice infected with the murine gamma herpesvirus MHV-68

Martin Häusler a,⁎, Bernd Sellhaus b, Simone Scheithauer c, Bärbel Gaida a,b, Sabrina Kurooka c, Katharina Siepman a,b, Anna Panek b, Wibke Berg a,c, Andreas Teubner d, Klaus Ritter c, Michael Kleines c

a Department of Pediatrics, University Hospital, RWTH Aachen, Pauwelsstr. 30, 52074 Aachen, Germany
b Department of Neuropathology, University Hospital, RWTH Aachen, Pauwelsstr. 30, 52074 Aachen, Germany
c Division of Virology/Institute of Medical Microbiology, University Hospital, RWTH Aachen, Pauwelsstr. 30, 52074 Aachen, Germany
d Institute of Laboratory Animal Science, University Hospital, RWTH Aachen, Pauwelsstr. 30, 52074 Aachen, Germany

Received 12 January 2007; received in revised form 30 May 2007; accepted 25 June 2007
Available online 4 July 2007

Time for primary review 25 days

Abstract

Objectives: Animal models of human Epstein–Barr virus (EBV) infection include EBV infection of primates and infection of mice with MHV-68, a further gamma herpesvirus (γ-HV). We aimed at extending the MHV-68 model to study γ-HV-related cardiac disease.

Methods: Newborn wild-type BALB/c- (n=107), wild-type C57BL/6- (n=17) and immunodeficient B6-(Rag1)™ mice (n=18) were infected by nasal inoculation and evaluated for histopathological changes as well as tissue viral loads.

Results: From day 5 on BALB/c mice showed myocardial viral replication. Whereas focal inflammation occurred simultaneously, necrosis was first observed 9 days post-infection. The maximum rates of necrosis (40%) and of focal inflammation (33%) were found after 10 to 12 and 33 to 35 days, respectively. Some animals developed persistent viral activity and inflammation throughout the observation period of three months. Inflammation was mainly related to T cell infiltrates. Although C57BL/6 mice also showed myocardial inflammation, necrosis was not found suggesting differences in the susceptibility to the virus in distinct mouse strains. In immunodeficient animals higher myocardial viral loads were observed compared to wild-type mice but no cardiac lesions, which suggests that the antiviral immune response contributed to the lesions.

Conclusions: The model system presented here is the first to allow detailed studies on cardiac disease caused by γ-HV infections and may facilitate the development of more specific treatment options for human cardiac EBV infection.

© 2007 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.

Keywords: Murine gammaherpesvirus 68; MHV-68; Epstein–Barr virus; Myocarditis; Animal model; Mouse

1. Introduction

Viral myocarditis and inflammatory dilative cardiomyopathy (DCM) are mainly related to enterovirus and adenovirus infections which, according to PCR studies on myocardial tissue of adults, account for 30%–50% of the patients. The Epstein–Barr virus (EBV), in contrast, has been found in up to 8% of the cases [1–7]. This rate of EBV infection may increase when evaluating more precisely defined groups of patients. Angelini et al., for example, detected EBV as causative agent in 2/7 patients with unexplained cardiac infarction [8]. In children EBV-related heart disease may be of higher clinical impact. Focusing on patients with primary EBV infection, Li et al. detected cardiac arrhythmia and myocarditis in 1.6% and 2.6% of the patients, respectively [9]. Performing myocardial PCR in patients with sudden infant death syndrome Dettmeyer et al. detected EBV in 3/62 cases (5%) [10]. According to Amabile et al., the prevalence of EBV in children with fulminant myocarditis may be similar to
the prevalence of enterovirus and parvovirus B19, being present in 2, 1 and 1 out of 11 patients, respectively [11].

Knowledge on the mechanisms of viral myocarditis is largely based on mouse models of enterovirus infection [6,12,13] showing a hematogeneous spread to the heart, first establishing viral replication in endothelial cells, then in myocardiocytes. Direct virus-related cell lysis occurs within 3 days after infection. From day 4 to 7 virus elimination is initiated by NK cell activity. Thereafter cytotoxic T cells, macrophage activity and neutralizing antibodies establish viral clearance within two weeks. Tissue lesions may also be caused by humoral and cellular immune mechanisms including a cellular immune response to viral antigens expressed on cardiac cells such as MHC-I complex-bound viral peptides or a humoral immune response to cardiac antigens cross reacting with viral antigens [14,15]. This inflammation is regulated by different chemokines and cytokines, including IL-1beta, TNF-alpha, IFN-gamma, IL-2 and NO [16]. Some animals develop persistent infection with transcription of viral genes without composition of viral particles. In enterovirus infection this affects myocytes and inflammatory cells (B cells) [13,14]. Viral persistence and the resulting immune response contribute to DCM.

The processes causing cardiac damage in EBV infection, in contrast, are uncertain which is mainly explained by the absence of an animal model [6,12]. MHV-68 infection of mice has been established as an animal model for certain aspects of human EBV infection [17–19]. MHV-68 and EBV are gamma herpesviruses sharing considerable homologies. Primary infection of mice with MHV-68 is clinically similar to human primary EBV infection and both viruses establish latent infection of B cells. According to sparse literature data, MHV-68 seems to spread hematogenously to the heart, showing maximum myocardiad replication between 5–10 days after infection followed by viral clearance within the following 20 days [17,18,20]. Classical histopathological signs of myocarditis related to MHV-68 infection have not yet been reported. We have recently shown for the first time that the MHV-68 mouse model mirrors human cerebral EBV infection [21]. Here we report that this model can be extended to study cardiac inflammation.

2. Methods

2.1. Animal housing and animal-related procedures

Immunocompetent BALB/c and C57BL/6 wild-type mice were purchased from Taconic (Bomholt, Denmark). T and B cell-deficient B6-(Rag1)™ mice from Jackson/Charles River Laboratories (Sulzfeld, Germany). Prior to viral inoculation the immunocompetent animals were housed in barrier rooms and regularly checked for pathogens. B6-(Rag1)™ mice were housed in isolator units. All infected mice were also housed in isolator units. Neonatal mice were separated from their mothers at three weeks of age. For infection 20 μl of sterile filtrated cell culture supernatant (2 × 10^5 plaque forming units of MHV-68) was applied on the mice’s noses and allowed to be inhaled. At predefined time points or immediately following the occurrence of severe clinical symptoms the animals were sacrificed by isoflurane anesthesia. The organs were removed, fixed in 4% paraformaldehyde and/or stored unfixed at −70 °C. The study was performed in accordance with the German laws on animal experiments (Tierschutzgesetz). It conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

2.2. Virus and cell culture

The adherent cell line BHK-21 was grown in Eagle Minimum Essential Medium containing 10% FCS supplemented with 2% antibiotics and 0.2% non-essential amino acids at 37 °C in a humidified atmosphere (5% CO2). MHV-68 was grown on BHK-21 cells. For virus preparation BHK-21 cells, grown to a coverage of 2/3 of a 50 ml tissue culture bottle, were inoculated with 1 ml of virus suspension containing 10^6 pfu of MHV-68 for 1 h. After addition of BHK-21 growth medium, the samples were cultured for further three to four days until complete cellular lysis was accomplished. The supernatant was harvested, centrifuged (400 g, 10 min), sterile filtrated, aliquoted, and stored at −70 °C. One aliquot of each preparation was quantified according to the Kärber method [22].

2.3. Quantification of viral loads by PCR

Using the primers MHV-68 G50a-5′ (5′-gat gga aac aga aaa cga gcc c-3′) and MHV-68 G50a-3′ (5′-ttg ctt gtt tct ggg gag gtt t-3′) a subgenomic fragment of the MHV-68-encoded gene G50 was amplified and subcloned into the vector pGEM3.1 using the T4 DNA ligase to create the resulting vector pGEM-MHV-G50. The vector pGEM-MHV-G50 was transformed into the E. coli Top10 (Invitrogen), the resulting vector pGEM-MHV-G50 was quantified by spectrophotometry and used as a quantification standard. For quantification of MHV-68 viral loads DNA was extracted from blood using the QIAamp DNA Blood Mini kit and from mouse tissue using the QIAamp DNA Mini kit for tissues (Qiagen, Hilden Germany). Homogenization of tissue was done using the Mixer Mill MM 300 (Retsch, Hach, Germany) (DNA: 30 Hz, 20–30 s RNA: 20 Hz, 2 × 1 min). DNA extracts were used as template in a real time PCR protocol with pGEM-MHV-G50 as quantification standard. An aliquot of 2 μl template was added to 18 μl of reaction mixture resulting in the final concentrations 4 μM MgCl2, 0.5 μM of primer MHV-68 G50b-5′ (5′-aaa agt tet gca tcc cag acc c-3′) and primer MHV-68 G50b-3′ (5′-agg gct aat ggg tga aaa tgg c-3′), each, and 10% LightCycler Faststart DNA SYBR green master mix. The experimental protocol was as follows: Denaturation 10′ 95 °C. Amplification 45 cycles of 0′ 95 °C, 10′ 66 °C and 12′ 72 °C (followed by a melting curve). Tissues shown to be MHV-
After blocking of endogenous peroxidase (3% H₂O₂) antigen-nized (xylol) and rehydrated (alcohol 100%, 97%, 70%). For immunohistochemistry the sections were first deparaffi-

## 2.4. Histopathology and immunohistochemistry

Staining for hematoxylin and eosin, Ladewig staining and immunohistochemistry were performed according to routine methods using 3 μm sections of paraffin-embedded tissue. For immunohistochemistry the sections were first deparaffi-

### 2.5. Immunofluorescence assay

Slices of mouse tissue were deparaffinized as described above. Antigen demasking was performed by cooking the slices for 20 min in a citrate-buffered solution consisting of citric acid (2.7 ml, 0.1 M), natrium citrate (12.3 ml, 0.1 M, pH 6.0) and bidistilled water (285 ml). After washing in phosphate buffered saline (PBS, 3 times for 5 min) the slices were incubated with rabbit anti-MHV-68 antibody (dilution 1:128) (30 min, 37 °C), kindly provided by Prof. A. Nash, Edinburgh, UK. After repeated washing with PBS the slices were incubated with FITC-conjugated swine anti-rabbit antibodies for 30 min at 37 °C (dilution 1:20, DAKO). Finally, the slices were washed three times in PBS and counterstained with hemalaun.

### 2.6. Statistical analysis

For statistical analysis the Wilcoxon rank sum tests for paired and non-paired samples, respectively, were used. For graphical analysis of paired quantitative viral loads the tissue viral load ratios (viral load tissue 1/viral load tissue 2) were calculated. To avoid division by zero, tissues with viral loads below the detection threshold were arbitrarily attributed the viral load of 1 genome equivalent (Geq./mg). Therefore, animals with higher viral loads in tissue 1 show values above 1 and vice versa. Animals with equal loads and animals with viral loads below the threshold in both tissues show values of 1.

## 3. Results

### 3.1. Histopathological findings in non-infected BALB/c mice

Myocardial necrosis, characterized by severe focal tissue damage with dissolution of cell boundaries, was not present in any of the 47 BALB/c control animals aged 3 to 94 days. On leukocyte (LCA) staining (n = 47) 20% of the animals showed no inflammatory cells, whereas sparse or prominent diffuse infiltrates were detected in 53% and 27%, respectively. Focal infiltrates characterized by accumulations of tightly adjacent inflammatory cells, in contrast, were completely absent. According to immunohistochemical studies for B cells (n = 14), T cells (n = 14), F 4/80 (monocytes; n = 22) and granzyme B (cytotoxic activity; n = 10), these diffuse leukocyte accumulations could be attributed to B cells. T cells were very sparse, F 4/80 antigen staining was always negative and granzyme B staining proved positive for epicardial cells in 80% of the mice whereas perivascular, diffuse parenchymatous or endocardial cells were stained very rarely. With regard to these data, necrosis and focal infiltrates were considered the most important criteria for defining pathological changes in mice infected with MHV-68.

### 3.2. Time course of cardiac MHV-68 infection in BALB/c mice

107 BALB/c mice infected within 24 h after birth and showing a positive MHV-68 viral load in at least one tissue...
(blood, liver, heart, brain and/or lung) were available for further analysis. Paired blood and heart PCR data were available from 74 of these animals. Comparison of these data revealed that cardiac viral loads exceeded blood viral loads in distinct animals beginning on day five after infection, suggesting intra-cardiac viral replication (Fig. 1). In many animals this difference in viral loads disappeared or decreased markedly within three weeks, whereas some showed persistently high myocardial viral loads throughout the observation period of 3 months, suggesting chronic viral activity. Absolute myocardial viral loads studied in 85 animals gained a maximum between 7 and 12 days post-infection and declined significantly thereafter, however remaining detectable during the maximum observation time in distinct animals (Fig. 2).

3.3. Time course of MHV-68 infection in further organs of BALB/c mice

Viral loads in lung, spleen, and liver tissues were found to exceed the viral loads in the corresponding blood samples after one, three, and three days, respectively, indicating that viral replication occurred earlier in these organs than in the hearts. Maximum median viral loads in these organs were log8, log7, and log3 Geq./mg tissue, respectively. These were detected five to 21 days after infection. Maximum median viral loads in the hearts, for comparison were log9 Geq./mg tissue. Only liver tissue showed a rapid viral clearance, leading to a median viral load of 0 Geq./mg tissue within three weeks. Spleen and lung tissue, in contrast, showed persistently high median viral loads. After three months, the values were log5 and log6 Geq./mg tissue, respectively. Paired pulmonary and cardiac MHV-68 PCR data were available from 65 animals. Animals with cardiac viral loads exceeding pulmonary viral loads were found between day 7 to 14 post-infection only, whereas pulmonary viral loads were equal to higher before and thereafter (Fig. 3). All these data comply with the fact that MHV-68 is replicated in the lung first, then in the heart, and finally it enters viral latency in splenic and pulmonary cells.

3.4. Detection of viral antigen in cardiac tissue of infected BALB/c mice

To evaluate whether positive PCR results were not due to otherwise inactive DNA we performed immunofluorescence testing for the presence of viral antigen in myocardial tissue. Positive staining of cardiomyocyte cytoplasm was found in
15 out of 25 animals studied (Fig. 4). This included 10/17 animals investigated 1–36 days after infection as well as 5/8 animals investigated at the age of 3 months.

3.5. Histopathological changes in myocardial tissue of infected BALB/c mice

Histopathological data were available from 92 randomly selected animals. In all these animals at least three slices deriving from the largest available heart diameter were stained for H&E and/or LCA. In contrast to controls, infected animals developed focal leukocyte infiltrates and myocardial necrosis (Figs. 5 and 6). First necroses (H&E staining) were observed 9 days after infection. The maximum rate of necrosis was detected 10–12 days post-infection. After that a decrease was observed. Focal leukocyte infiltrates were already present before the occurrence of necroses. Until day 33 post-infection the prevalence of focal infiltrates increased. In distinct animals these persisted throughout the observation period of 3 months. DCM as assessed by histopathological studies was not found in any animal.

Cardiac necrosis was frequently associated with clinical symptoms such as dystrophy and irritability. In consequence, 9 of the animals with necrosis had been removed prior to the intended date. In contrast, none of the animals without necrosis (including animals with focal infiltrates and animals without cardiac findings) had to be removed early. This may have biased the age distribution of the lesions displayed in Fig. 6.

Further specific staining procedures were performed on randomly selected animals showing abnormal findings on LCA staining. In contrast to non-infected controls in which interstitial B cells were the predominant leukocyte type, no correlation between leukocyte and B cell staining was found.

Fig. 5. Cardiac histopathology of BALB/c mice infected with MHV-68. A: multiple myocardial necroses (*) (Ladewig staining, age 9 days, bar=550 μm). B: focal (arrow) and diffuse (*) myocardial leukocyte infiltrates (leukocyte staining, age 10 days, bar=50 μm). C: transmural infarction with central calcification (arrow) surrounded by a fibrous wall (*) (H&E staining, age 3 months, bar=150 μm).

Fig. 6. Development of cardiac inflammation and necrosis in 92 BALB/c mice infected with MHV-68 over time according to H&E and leukocyte staining. The highest rate of tissue damage was found between 10–12 days after infection. Both, focal inflammation and necrosis can be observed.
in infected animals that also showed sparse diffuse B cells only. In contrast, the pattern of T cell infiltrates correlated with the pattern of leukocyte staining which indicates that inflammation was mainly caused by T cells. As in control animals, staining for F4/80 was negative in all 24 animals studied. Throughout all age groups of infected animals the distribution of granzyme B-positive cells was comparable to controls, with only few animals showing small focal infiltrates. No accumulation of granzyme B-positive cells was found in necrotic areas. Importantly, positive F4/80 and granzyme B staining was detected in the lungs of the same animals which suggests an organ-specific immune response.

3.6. Comparison of viral loads and histopathological findings in infected BALB/c mice

Comparison of myocardial viral loads between animals with (n=12) or without (n=50) necrosis revealed significantly higher viral loads in animals with cardiac lesions (Fig. 7) (p<0.0001).

3.7. Virological and histopathological findings in infected C57BL/6 mice

Considering the strain-specific differences of myocardial damage after enterovirus infection we studied 17 newborn C57BL/6 mice at the age of 14 to 21 days. None of these animals developed cardiac necrosis. This contrasts 12 randomly selected age-matched BALB/c mice which showed similar cardiac viral loads but included four with cardiac necrosis.

3.8. Virological and histopathological findings in infected immunodeficient B6-(Rag1)™ mice

To study whether an impaired immune defense increases the risk for myocardial damage, 18 neonatally infected B- and T cell-deficient B6-(Rag1)™ mice were studied between 14 and 21 days of age. B6-(Rag1)™ mice share the genetic background with C57BL/6 mice. B6-(Rag1)™ mice developed markedly higher viral loads in blood (p<0.001) and cardiac tissue (p<0.01) compared to C57BL/6 mice. In addition, within B6-(Rag1)™ mice the cardiac viral loads significantly exceeded the blood viral loads, indicating intramyocardial viral replication (p<0.001, Fig. 8). However, no myocardial necrosis was found in any of these animals.

3.9. Clinical findings in BALB/c, C57BL/6 and B6-(Rag1)™ mice infected with MHV-68

None of the C57BL/6 or the B6-(Rag1)™ mice showed clinical symptoms or died spontaneously during the maximum follow-up period of 21 days. As already mentioned above, this contrasts the findings in BALB/c mice. According to Kaplan–Meier analysis, 9% of the BALB/c mice can be expected to die spontaneously and 18% can be expected to die or to show severe clinical symptoms until 21 days of age.

3.10. Detection of autoantibodies to cardiac tissue in BALB/c mice

Sera of four non-infected control animals as well of 8 neonatally infected mice that had been sacrificed 14 to 35 days after infection were studied for the presence of autoantibodies to cardiac tissue. The sera were incubated with heart tissue slices deriving from the same animals as well as with tissue slices deriving from further animals. A positive reaction was only found when incubating sera of infected animals with tissue slices deriving from the same animals. It was found in 5 out of 8 animals and was always characterized by a nuclear staining pattern. Sera of non-infected control animals, in contrast, always proved negative.
4. Discussion

Our findings establish the MHV-68 mouse model as a new tool to study cardiac gamma-herpesvirus infections. Myocardial viral replication was initially detected five days after infection, in contrast to pulmonary, splenic and hepatic viral replication that occurred within 24 to 72 h. The maximum median viral loads in cardiac tissue were comparable to those observed in the lungs and spleens (log7–log9 Geq./mg tissue). Focal cardiac infiltrates without necrosis were detected simultaneously with viral replication, necrotic lesions occurred four days later. The rate of animals with high myocardial viral loads, focal infiltrates and necrosis markedly decreased after 3 weeks which can in part be explained by the fact that animals with cardiac necrosis frequently showed signs of severe clinical disease leading to early removal. Our findings comply with the literature on MHV-68 infection, suggesting maximum myocardial viral replication between 5 days and 3 weeks after infection [17,18,20]. Myocardial inflammation, interestingly, did not disappear completely with time after infection. In some animals it persisted throughout the maximum observation period of three months, as did high viral loads and viral antigen, suggesting a chronic process.

In accordance with enterovirus myocarditis, T cells were the main leukocyte subset detectable in the hearts 2 to 3 weeks after inoculation [12,13]. Monocyte infiltrates, surprisingly, were not found in the hearts of MHV-68-infected animals as were granzyme B-positive cells. This differs from enterovirus infection but complies with the findings of Usherwood et al. [23] showing that control of MHV-68 virus infection is independent from NK cell activity. However, monocytes and granzyme B-positive cells were detected in the lungs of the same mice. This indicates that the immune response to MHV-68 is variable in an organ-specific manner and differs from the immune response to further cardiotropic viruses. Eventually organ-specific differences in the immune response might contribute to the different susceptibility of distinct tissues to MHV-68 infection which should be studied more intensively.

Kawai et al. suggested that clearance of enteroviruses from the myocardium may also depend on B cell activity as T cell-depleted mice have been found to show considerably less myocardial damage [12]. According to this hypothesis, T cells would predominantly account for immune-mediated damage. Our findings comply with this hypothesis. In non-infected controls, B cells were the predominant leukocyte type resident in myocardial tissue suggesting a major impact of B cells for cardiac immune surveillance in mice. B- and T cell-deficient B6-(Rag1)TM mice, in turn, showed very high myocardial viral loads but no myocardial necrosis. Therefore viral activity is not sufficient to explain myocardial damage. A complex interaction between viral activity and the immune response reducing viral loads as well as causing tissue damage has to be assumed. This assumption is supported by our studies on C57BL/6 wild-type animals. In contrast to BALB/c mice, these C57BL/6 mice did not show myocardial necrosis after MHV-68 infection, although focal and diffuse infiltrates were observed. This complies with the fact that different mouse strains respond differently to enterovirus infection with BALB/c mice showing more myocardial damage than C57BL/6 mice [13]. Differences in major histocompatibility complexes have been suggested to affect the specific immune response or the susceptibility to autoimmune processes. Among the animals infected with MHV-68 strain-specific differences were not only related to cardiac damage but also affected the clinical outcome as MHV-68-related morbidity and mortality were also restricted to BALB/c animals. Even in BALB/c mice the rate of severe myocardial changes (up to 50%) and of severe clinical disease (18% after 21 days) seems not very high; an increased number of mice will be required when studying the efficiency of different treatment options. On the other hand, this rate of animals affected by MHV-68 infection may facilitate to evaluate the impact of risk factors that enhance the occurrence of myocardial lesions.

Based on our histopathological investigations, none of the infected BALB/c mice developed DCM within the maximum follow-up period of three months. Eventually DCM would occur after longer observation periods. Even three months after infection, a considerable number of mice showed persistent inflammation, persistently high PCR-proven viral loads or were found positive for viral antigen staining in myocardial tissue. According to further animal models of viral cardiac infection these findings are important preconditions for the development of inflammatory heart dilation [12,13] (Figs. 2 and 6).

It has previously been shown that MHV-68 infection is associated with a polyclonal B cell stimulation with synthesis of autoantibodies to various antigens [24]. It might be speculated whether this includes the production of antimyosin autoantibodies which contribute to myocardial damage. Our findings do not support this hypothesis. On the one hand a positive nuclear staining pattern was found repeatedly which confirmed an MHV-68-related synthesis of autoantibodies. On the other hand, in case of autoantibodies to myosin a cytoplasmic staining pattern would be expected which, however, was not detected.

Myocarditis has also been reported in mice infected with further herpesviruses including herpes simplex virus (HSV) and cytomegalovirus (CMV). Similar [25,26] to MHV-68-infected mice these animals developed acute and chronic myocarditis with persistent cardiac viral activity and inflammation. BALB/c mice also proved more susceptible than C57BL/6 animals. These different models, however, are not interchangeable. For example, infection of newborn BALB/c mice with HSV-1 is lethal for all animals. Mice infected at older age develop myocarditis with Cowdry A inclusion bodies present in cardiac tissue in the acute and chronic stage. Cytomegalovirus myocarditis, in turn, is not dependent on neonatal infection but can also be induced in six-week-old animals. This suggests that apart from host-related factors
virus-related factors strongly influence the extent of tissue damage even within the family of herpesviridae.

In summary, the MHV-68 mouse model seems promising for studying acute and chronic gamma-herpesvirus myocarditis which will allow addressing of important questions concerning organ- and agent-specific characteristics of the immune response, the impact of chronic myocardial cell and B cell infection as well as of humoral and cell-mediated autoimmunity. This might facilitate to establish specific treatment strategies for patients with distinct viral diseases, including cardiac gamma-herpesvirus infections.

References


