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# Persistence of virus and viral genome in myocardium after Coxsackievirus B3-induced murine myocarditis

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## SUMMARY

Following infection with Coxsackievirus B3 (CVB3), A-strain mice develop ongoing myocarditis that persists after the virus ceases to be cultivatable from heart tissue. We studied the natural history of this virus-induced but apparently autoimmune inflammation by means of *in situ* hybridization (ISH) and by polymerase chain reaction (PCR). Both ISH and culture allowed detection of virus up to 2 weeks post-infection in virtually all heart tissues. In contrast, PCR revealed the presence of viral genome for a substantially longer period of time, i.e. at least 34 days after CVB3 infection. Similarly, the majority of mice showed myocardial inflammation at this time point. However, the persistence of virus did not correlate with ongoing myocarditis, and *vice versa*. Most mice with ongoing myocarditis produced heart myosin autoantibodies, most probably as a result of tissue damage. The lack of correlation between presence of ongoing inflammation and persistence of virus supports our previous view that the late phase of CVB3-induced myocarditis is mediated by autoimmunological mechanisms.

**Keywords** autoimmunity polymerase chain reaction *in situ* hybridization *Coxsackievirus B3* myocarditis

## INTRODUCTION

Enteroviruses are frequently associated with inflammatory heart disease. In particular, Coxsackieviruses are believed to be one primary etiologic agent. In most cases, the virus is eliminated from the blood and the tissues by humoral and cellular effector mechanisms, thereby allowing the individual to recover relatively rapidly. Under certain conditions, however, the acute phase of the disease is followed by an ongoing inflammatory process, even though the initiating virus can no longer be cultured from the tissue. A variety of clinical and experimental data suggest that the later stages of myocarditis are mediated by autoimmune mechanisms [1–4].

The most frequently used experimental systems to study inflammatory heart diseases and post-infectious autoimmunity are murine models of picornavirus-induced myocarditis and encephalomyocarditis. For instance, a few days after infection with Coxsackievirus B3 (CVB3), *H-2* congenic mouse strains on the A and B.10 background develop an acute form of myocarditis which is due to the virus-mediated myocyte damage and the ensuing inflammatory response [5]. While the B.10 *H-2* congenics generally recover rapidly, mice on the A background (e.g. A/J, A.SW, A.CA) develop persistent myocarditis characterized by a diffuse interstitial cell infiltrate and by the presence of heart myosin-specific autoantibodies [6,7]. However, virus cannot be cultured from the heart tissues later than 9 days after the infection [5]. Based on these observations, the late phase of the disease seems to be self-sustaining and possibly autoimmune in nature. However, the technique of culturing virus in an indicator cell line will detect only infectious virus, and might not be sufficiently sensitive. Therefore, we have examined the heart tissues for the presence of viral genome by using *in situ* hybridization (ISH) and polymerase chain reaction (PCR) techniques.

On the assumption that the late phase of myocarditis is mediated by persisting virus or viral genome, one would expect a good correlation between presence of an inflammatory infiltrate and presence of virus.

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## MATERIALS AND METHODS

### *Animals*

The breeding stocks of A/J and A.SW/SnJ mice were originally purchased from Jackson Labs (Bar Harbour, ME), and were raised at the Institute of Virology and Immunobiology, University of Würzburg. Infected animals were kept with their foster mothers until 4 weeks of age.

*Infection*
Fifteen-day-old mice were infected intraperitoneally with $10^5$ $TCID_{50}$ of CVB3 (VR-30; ATCC, Bethesda, MD) [5] and killed at various time points by cervical dislocation. One portion of tissue samples was snap frozen in liquid nitrogen for later viral culture, RNA extraction and immunohistochemistry. The other portion was fixed in 10% buffered formalin and then paraffin-embedded for *in situ* hybridization and histopathologic examination. Sera were obtained by retroorbital bleeding at the time of sacrifice.

*Histopathology*
Transverse paraffin sections were obtained at eight different levels and were examined for cellular infiltration and myocyte necrosis. The severity of myocarditis was graded according to a previously described scoring system [5]: 0, no infiltration; 1, up to 5% of the histologic cross section infiltrated; 2, 5–10%; 3, 10–20%; 4, >20%.

*Myosin autoantibodies*
Myosin IgG autoantibody titres were determined in an ELISA in 96-well microtitre plates by using the cardiac myosin isoform as test antigen [7]. Titres below 1/40 were taken as negative, as these values might reflect the presence of 'natural' antibodies [7].

*Detection of cultivatable virus*
Myocardial tissues were individually analysed for the presence of infectious virus by adding supernatants from homogenized hearts to confluent cultures of Vero monkey kidney cells as described [5]. Results were taken as positive when a cytopathic effect was seen within 2 weeks.

*Preparation of probes*
The CVB3 cDNA clone pCB3-M1 (kindly provided by Dr R. Kandolf, Martinsried, Germany) [8] was digested with KpnI, HindIII and EcoRI to generate a 684-bp-long fragment from the region coding for viral proteins which is highly specific for CVB3. By digestion with KpnI and PstI a 464-bp-long fragment from the 5′-non-coding region was obtained, which is highly conserved among enteroviruses. These fragments were inserted into the T7 promoter-containing pspT18 and pspT19 vectors (Boehringer, Mannheim, Germany) at the polylinker sequence site. *In vitro* transcription of the inserts with T7 RNA polymerase produced sense or antisense RNA depending on the orientation of the insert in the plasmid. Each strand was radioactively labelled with $^{35}$S-UTP (Amersham, Aylesbury, UK; specific activity 1000 Ci/mmol) during the transcription reaction, resulting in an average activity of $3 \times 10^7$ ct/min per probe.

*ISH*
ISH was carried out using a modification of the method of Höfler *et al.* [9]. Briefly, formalin-fixed, paraffin-embedded tissues were sectioned and deparaffinized by standard methods. The sections were then hybridized overnight at 43°C in probe solution (50% deionized formamide; 10% dextrane sulphate; 20 mM HEPES pH 7·05; 1·2 M NaCl; 2 mM EDTA; 0·04% Ficoll, polyvinyl pyrrolidone (PVP), bovine serum albumin (BSA); 250 μg/ml tRNA; 100 mM dithiothreitol (DTT); 1 U/μl RNAse inhibitor (RNAsin; Promega, Madison, WI)) containing $10^5$ ct/min per μl of probe. The next day the slides were washed with 2 × SSC for 15 min at room temperature and 2 × SSC for 15 min at 37°C. RNA digestion was carried out with 10 μg/ml RNAse A and 1 U/ml RNAse T in 2 × SSC for 30 min at 37°C. The slides were then washed with 0·1 × SSC for 10 min at 50°C and for another 10 min at room temperature. The slides were dehydrated in graded ethanols and air dried. Autoradiography was performed with Ilford K2 emulsion; a minimum exposure time of 3 weeks at 4°C was strictly observed. Infected Vero monkey kidney cells served as positive controls. As a negative control, each section was hybridized with plasmid-derived RNA.

*PCR and Southern blotting*
Total RNA was extracted from specimens by guanidinium thiocyanate phenol-chloroform, precipitated with isopropanol and washed in 70% ethanol [10]. The RNA pellet was dissolved in 20 μl of twice distilled water (treated with diethyl-pyrocarbonate) and aliquoted. Ten microlitres of reverse transcriptase buffer (50 mM Tris-HCl pH 8·3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$) containing 10 mM dNTP, 20 U RNAsin, 100 pM random hexamers and 200 U M-MLV reverse transcriptase (Bethesda Research Laboratories) were added to each 10-μl sample. Oligonucleotide downstream primers for CVB3 were added to a final concentration of 0·1 pM [11]. The reaction was carried out at 37°C for 60 min and was stopped by heating the samples for 5 min at 95°C.

Eighty microlitres of PCR reaction mixture consisting of 50 mM KCl, 10 mM Tris-HCl pH 8·3, 1·5 mM $MgCl_2$, 0·0001% (w/v) gelatine, 1 U Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) and upstream and downstream primers (final concentration 0·1 pM) were added to the c-DNA sample. DNA amplification was carried out in a Bio-Med thermocycler (Bio-Med, Theres, Germany) for 30 cycles. Cycle duration and temperatures were optimized (denaturation 94°C, 1 min; annealing 58°C, 2 min; extension 72°C, 3 min). A primer pair of B1 (map position 1353–1372; 5′-AACGCTAGACAACACCC-CATC-3′) and B2 (map position 1423–1443; complementary strand: 5′-GTTGGACCCGGATGCGACCGG-3′) was chosen amplifying a 91 base pair sequence within the capsid protein coding region unique for CVB3 genomes (Fig. 1a) [12,13].

To prove that the bands detected were indeed viral the amplification products were analysed by Southern blotting using a CVB3-specific probe sequence (Fig. 1b). A 20-μl aliquot of the amplification product was fractionated by 3% NuSieve agarose (FMC Bioproducts, Rockland, ME) gel electrophoresis containing ethidium bromide (0·5 μg/ml) in TBE (90 mM Tris pH 8·3, 90 mM boric acid, 2·5 mM EDTA). The DNA pattern was documented photographically at 302 nm. Southern blot analysis was performed by blotting gels to nylon membranes (0·2 μm; Schleicher & Schüll, Dassel, Germany) according to standard techniques [14,15]. Radiolabelled probes were prepared by 5′-end labelling (Clontech 5′-end DNA labelling kit) [14]. Filters were prehybridized (10 × Denhardt's solution (0·2% Ficoll, 0·2% BSA, 0·2% PVP), 10% dextran sulphate, 1% SDS, 3 × SSC, 50 mg/l salmon sperm DNA) for 3 h and incubated with the 5′-$\gamma^{32}$ P-ATP-radiolabelled probe B3 5′-TTGCTGGGGGGCGATACGGCAAAGGAGTTT-3′ (specific activity approximately $1 \times 10^7$ ct/min per μg) at a final concentration of 1 pmol/ml for 4 h at 65°C. Filters were washed

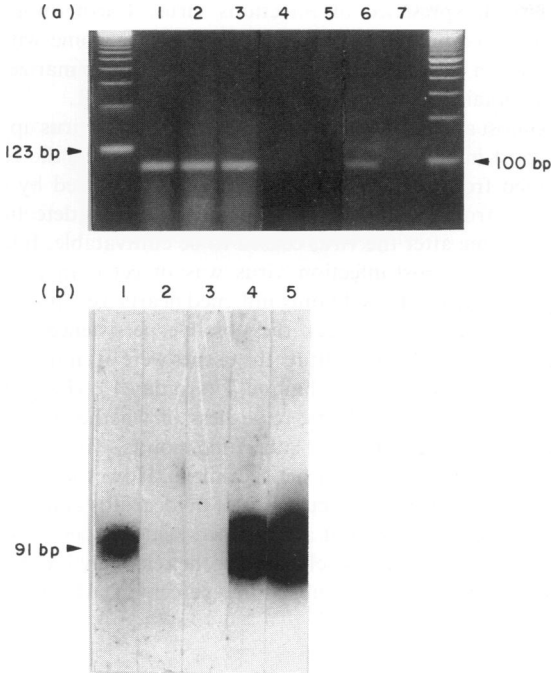


**Fig. 1.** (a) Amplification products of Coxsackievirus B3 (CVB3)-infected mouse hearts (lanes 1–3) and CVB3-infected Vero cells (lane 6). The amplification with primer pair B1/B2 shows the expected 91-bp signal. Lanes 4 and 5, hearts from infected mice negative for CVB3 virus; lane 7, uninfected Vero cells. Polymerase chain reaction (PCR) products were electrophoresed in a 3% Nu Sieve gel and stained with ethidium-bromide; 123-bp and 100-bp ladders (BRL) were used. (b) Southern blot hybridization of PCR products from CVB3-infected mouse hearts (lane 1, 4, 5). Lane 2, uninfected Vero cells; lane 3, Vero cells infected with parainfluenza viruses.

at 60°C with several changes of 3 × SSC, 0·1% SDS and 1 × SSC, 0·1% SDS, and exposed to Kodak X-AR S film for 2–24 h.

The absence of Taq polymerase inhibitors and sufficient transcription of c-DNA was proven by PCR of an aliquoted RNA sample with β-actin primers. The chosen oligonucleotides A1 5′-ATGGATGATGATATCGCCGCG-3′ and A2 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGGGCC-3′ (Clontech) amplify a 1126-bp sequence of the β-actin gene [16]. Heart tissue samples were scored as negative if the fragment specific for β-actin but no fragment of CVB3 could be amplified.

RNA extraction, amplification of c-DNA and analysis of PCR products were carried out in different rooms, and all reagents were assayed for the presence of CVB3 RNA; positive and negative samples were tested in the same experiments to avoid false positive results.

To test the sensitivity of the assay, Vero cells were infected with CVB3 ranging from 10 plaque-forming units (PFU) down to $10^{-3}$ PFU. Cells were harvested 48 h after infection for CVB3-RNA extraction. Amplification of c-DNA of Vero cells infected with at least $10^{-1}$ PFU gave the expected 91 bp (CVB3) signals. Southern blot hybridization increased the sensitivity of the test by the factor 10 (data not shown). Thus, $10^{-2}$ PFU could be detected, which is approximately equivalent to 10 virions [17]. The specificity of the assay was confirmed, as neither amplification of Vero cells infected with 10 PFU of parainfluenza virus nor uninfected Vero cells yielded any positive signal.

## RESULTS

### *Myocardial lesions*

As shown in Table 1, myocarditis was detected in most mice during the early phase of the disease, i.e. up to day 13 after CVB3 infection. The lesions were focal in nature and were characterized by myocyte necrosis and a cellular infiltrate (Fig. 2a). Calcifications were rare or absent. Compared with the early phase, hearts obtained at later time points, i.e. after day 13 post-infection, showed a diffuse interstitial cell infiltrate (Fig. 2b). Twenty-eight days post-infection, the histologic picture was characterized by calcifications, scar formations and an extensive interstitial infiltrate (not shown). The increasing severity of myocarditis was observed particularly in the A.SW strain (Table 1).

### *Myosin autoantibodies*

Previous work has demonstrated the presence of heart-specific myosin IgG autoantibodies in A strain mice during the late phase of CVB3 infection [7]. The mice in the present study were therefore tested for autoantibodies to this antigen in an ELISA. As shown in Table 1, myosin autoantibodies were not detected before day 16 in A/J or day 21 in A.SW mice. Later, these antibodies were found in similar frequencies in both strains at an

**Table 1.** Myocarditis and myosin autoantibodies in CVB3-infected mice

| | | Myocarditis | | Myosin IgG autoantibody | |
|---|---|---|---|---|---|
| Strain | Day | Prevalence (positive/total) | Severity in positive animals | Prevalence* (positive/total) | $Log_2$ titre in positive animals |
| A/J | 6 | 7/9 | 1·3±0·5 | 0/9 | |
| | 13 | 2/3 | 1·5±0·7 | 0/3 | |
| | 16 | 4/6 | 1·3±0·5 | 2/6 | 7·3+1·4 |
| | 34 | 6/9 | 1·0±0 | 4/9 | 7·3+2·7 |
| A.SW | 6 | 2/2 | 1·0±0 | 0/2 | |
| | 21 | 16/18 | 2·0±0·7 | 13/18 | 7·0+1·7 |
| | 28 | 6/6 | 1·7±0·5 | 3/6 | 6·3+1·0 |

* Positive is equivalent to an autoantibody titre of greater than 1/20, i.e. $log_2$ titre > 4·3.

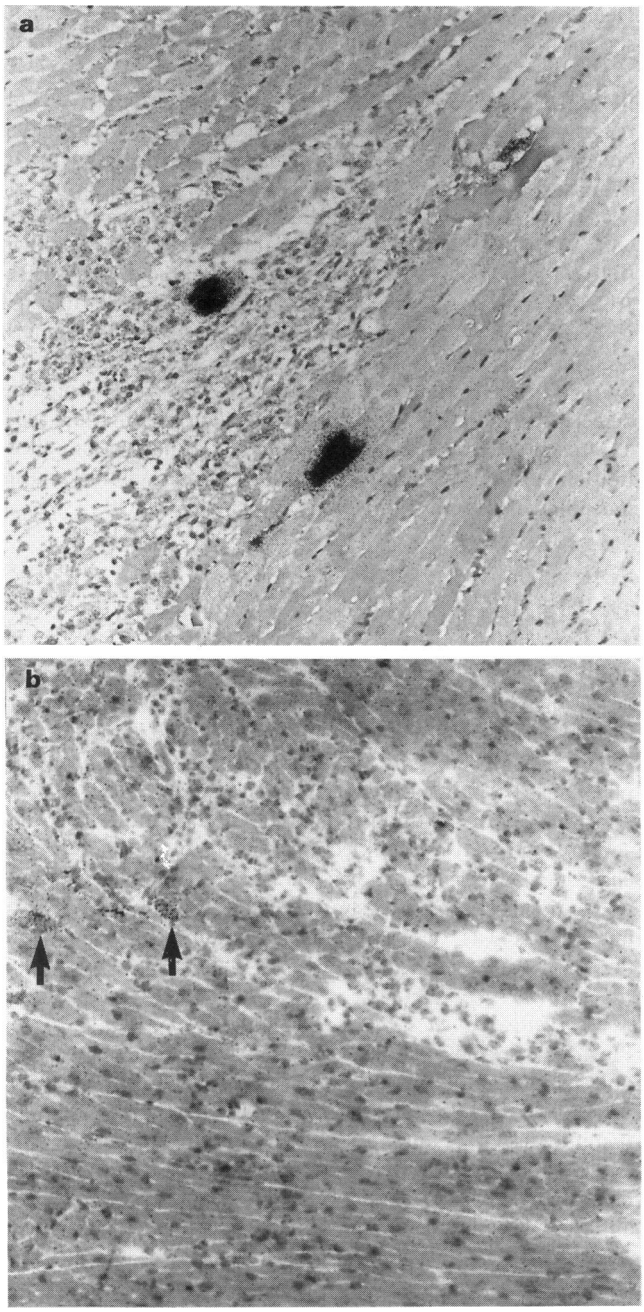


**Fig. 2.** Myocarditis and virus detection in heart tissues of Coxsackievirus B3 (CVB3)-infected A/J mice by *in situ* hybridization (ISH). (a) Focal cellular infiltrate and positive ISH signals 6 days after infection. The signals are located along muscle fibres and adjacent inflammatory cells. (b) By day 13, almost no *in situ* hybridization signals (arrows) are detectable. Note the presence of the diffuse interstitial cellular infiltrate (haematoxylin–eosin, ×250).

average titre of 1/160. In agreement with previous data [7], increased myosin autoantibody titres were found only in those mice which developed myocarditis.

*Viral persistence by culture, ISH and PCR*

The presence of virus or viral genome was determined by using a panel of parallel assays performed on one and the same tissue, as we wanted to investigate whether the presence of viral genome outlasts the presence of infectious virus. Furthermore, we wished to compare the localization of viral genome with the localization of myocardial lesions. Table 2 summarizes the results obtained from viral cultures, ISH and PCR.

Almost all heart tissues contained cultivatable virus up to 13 days post-infection, but not thereafter. Moreover, the results obtained from viral cultures were exactly paralleled by those obtained from ISH. This technique did not allow detection of viral genome after the virus ceased to be cultivatable. Interestingly, 6 days post-infection virus was detected in all heart tissues, whereas on day 13 only inflamed hearts were positive by ISH and culture. To check the possible persistence of virus undetectable by ISH or culture, the tissues were examined by the far more sensitive PCR technique. Up to day 13, viral genome was detectable in all hearts, regardless of whether the tissues were inflamed or not. At later time points, virus was still detectable, but not in all heart specimens. However, there was no correlation between occurrence of myocarditis and presence of viral genome, since not all inflamed heart tissues contained viral genome, and conversely, among the relatively low number of uninflamed tissues a substantial percentage still yielded a positive PCR signal.

*Localization of virus*

We examined the relationship between localization of viral genome detectable by ISH and inflammation on one and the same tissue section. On day 6, hybridization signals were mainly located along muscle fibres in the neighbourhood of the inflammatory infiltrate. Virtually no signals were seen exclusively within inflammatory cells (Fig. 2a). The appearance of the interstitial cellular infiltrate at later time points (Fig. 2b) correlated with the disappearance of the ISH signals.

## DISCUSSION

With the development of sensitive detection assays for viral genomes based on nucleic acid hybridization and PCR, it is now possible to screen infected tissues for viral sequences which could well persist beyond the ability to detect infectious virus by culture. This might be particularly important for more detailed investigations of whether chronic inflammations initiated by viruses are influenced by persisting viral RNA or DNA.

In the present study, the virus ceased to be cultivatable after day 13, a time point which is not substantially different from that observed previously [5]. In our test system, ISH was no more sensitive than *in vitro* cultures, even with the use of RNA probes. The technique of ISH has been used for the follow up of viral persistence in other murine models of CVB3 infection. For instance, in hearts of athymic NMRI mice viral genome could be detected for a substantially longer period of time compared with A/J and A.SW mice used in the present study [18]. However, it is likely that athymic mice did not eliminate virus as efficiently as immunocompetent animals. The natural history of other picornavirus infections seems to be similar to that observed with CVB3. As shown by Cronin *et al.* [19], encephalomyocarditis virus could be cultured from heart tissues of infected BALB/c mice for up to 2 weeks, but viral genome was detectable by ISH for an additional week or two. The myocardial inflammation caused by this virus persisted for at least 12 weeks, and is most probably autoimmunologically mediated. Recently, two groups of investigators independently studied the persistence of ence-

**Table 2.** Virus detectable by culture, ISH and PCR in heart tissues of CVB3-infected mice*

| Strain | Day | Culture† (positive/total) | *In situ* hybridization | PCR | |
|---|---|---|---|---|---|
| | | | | Myocarditis | No myocarditis |
| A/J | 6 | 9/9 | 9/9 | 7/7 | 2/2 |
| | 13 | 2/3 | 2/3 | 2/2 | 1/1 |
| | 16 | 0/6 | 0/6 | 3/4 | 1/2 |
| | 34 | 0/9 | 0/9 | 3/6 | 0/3 |
| A.SW | 6 | 2/2 | 2/2 | 2/2 | — |
| | 21 | 0/18 | 0/18 | 14/16 | 1/2 |
| | 28 | 0/6 | 0/6 | 6/6 | — |

* Data are expressed as number of positive animals per total number tested in each group.
† Positive is equivalent to a cytopathic effect within 14 days of culture (for details see Materials and Methods).

phalomyocarditis virus in DBA/2 mice by means of PCR [20,21]. Viral genome could be detected for at least 90 days post-infection. At this time point, however, the cellular heart infiltrate had disappeared, and myocardial fibrosis was prominent.

In the present study, the use of PCR also allowed the detection of viral genome for a much longer period of time compared with ISH and viral culture. This finding is not surprising, as PCR allowed the detection of as few as 10 virions per tissue sample. Our results suggest that ongoing myocarditis and persisting viral genome expression occur independently from each other. For this reason we believe that the late phase of the disease is not triggered by the virus, but is self-sustaining and is mediated by autoimmunological mechanisms. We cannot entirely exclude that persisting viral genome has some influence on the development of ongoing heart disease. However, this possibility is less likely, as during the late phase of the disease virus was detectable by PCR only. Thus, the amount of persisting virus must be very little. This observation further suggests that the mice effectively, although not entirely, cleared the virus during the first 2 weeks post-infection.

Recently, nucleic acid hybridization techniques to detect the presence of viral genome have been used increasingly to investigate endomyocardial biopsies obtained from patients [22–24]. Coxsackievirus RNA was not only detectable at an early stage of myocarditis, but also in chronic dilated cardiomyopathy, without attracting a cellular immune response. Moreover, viral RNA was found in the myocardium after the inflammation had healed, a situation similar to that found in our murine model. In agreement with Keeling *et al.* [25], our results suggest that persistent viral genome detected in endomyocardial biopsies does not have pathogenic or prognostic importance. A report by Cunningham *et al.* [26] on enteroviral RNA in muscle biopsies suggests that enterovirus persistence is due to a defect in control of viral RNA synthesis.

As already mentioned, our study provides indirect evidence for autoimmunological mechanisms acting in the late phase of viral myocarditis in A strain mice. We believe that the autoimmune response in these mice is directed to cardiac myosin released or exposed after virus-mediated myocyte damage. This hypothesis is strongly supported by the finding that myocarditis can be induced with purified cardiac myosin in genetically predisposed mice [27].

In agreement with previous results [7], most CVB3-infected mice produced autoantibodies to cardiac myosin during the late, but not during the early phase of the disease. This is similar to the situation observed in cardiac myosin-induced autoimmune myocarditis, where myosin autoantibodies are elicited as a consequence of the T cell-mediated heart muscle damage and the ensuing inflammatory response [28,29]. These autoantibodies are not pathogenic [28,30], and do not correlate with persistent infection, but can be used as a serologic marker for myocarditis in both CVB3- and myosin-induced disease [7,27]. As demonstrated earlier, molecular mimicry is an unlikely reason for the production of myosin autoantibodies in CVB3-infected A strain mice [31], although there seems to be some epitope similarity between enteroviruses and myosin [32].

Our results support the concept that the late phase of the viral disease is based on autoimmunity. Currently, detailed studies with cardiac myosin-specific T cells are underway to analyse the mechanisms of target recognition and destruction in the affected heart tissue.

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