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Coxsackievirus B3-Induced Myocarditis

Characterization of Stable Attenuated Variants that Protect Against Infection with the Cardiovirulent Wild-Type Strain

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Coxsackievirus B3 (CVB3) is the enterovirus most frequently involved in human myocarditis or dilated cardiomyopathy. Attenuated variants were derived from a cardiovirulent CVB3 reactivated from a sequenced, full-length cDNA clone. The prophylactic potential of these variants was assessed in SWR/Ola (H-2q) mice. Animals immunized with attenuated variants of CVB3 were protected from myocarditis when challenged subsequently with the cardiovirulent wild-type virus. In contrast to nonimmunized controls, the wild-type virus was not isolated from myocardium of protected mice, nor was viral RNA detected in myocardium by reverse transcription nested polymerase chain reaction. Specific antibody to CVB3 was demonstrated by virus neutralization assay and by indirect immunofluorescence. The attenuated phenotype of one variant, p14V-1, remained stable throughout 20 consecutive passages in SWR mice and induced a markedly lower level of autoantibody against mouse cardiac myosin heavy chain than the cardiovirulent wild type. These data demonstrate that attenuated strains protect against CVB3-induced myocarditis in mice, that the attenuated phenotype is stable, and that they do not persist in myocardium nor induce a significant level of anti-heart antibody against myosin heavy chain. These attenuants may be the basis of a live vaccine against CVB3 in the prevention of enteroviral heart muscle disease. (Am J Pathol 1997, 150:2197–2207)

Approximately 70 serotypes of human enteroviruses are known and are etiological agents of a wide spectrum of disease, ranging from disease of heart or skeletal muscle and the central nervous system to enteric or upper respiratory infection.[1] These viruses are ubiquitous pathogens, and although most enterovirus infections are subclinical, the high infection rates generate significant numbers of cases of clinically severe disease. Enteroviruses, particularly Coxsackie B viruses (CVB), are the commonest etiological agents of viral myocarditis and are associated with the subsequent development of dilated cardiomyopathy (DCM), demonstrated initially by virological or serological studies[2,3] and later by molecular biology techniques including reverse transcription nested polymerase chain reaction (RT-NPCR).[4–11] Enterovirus RNA can persist in myocardium until end-stage disease requiring cardiac transplantation[12] and is an independent predictor of poor prognosis.[13] In a prospective study involving 120 patients with myocarditis or DCM, enteroviral RNA sequences were detectable in 41 (34%) cases. Follow-up study shows that patients in the viral RNA-positive group had a poorer prognosis than the viral RNA-negative group.[14] These viruses are also implicated in skeletal myopathies[15,16] and in insulin-dependent diabetes mellitus.[17,18] Until recently, lack of information on which serotypes are

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most frequently associated with particular diseases has delayed the development of vaccines, in contrast to effective attenuated live vaccines available against poliovirus serotypes 1 to 3.

Identification of RT-PCR products of enteroviral RNA was carried out previously by several laboratories using group- or type-specific PCR primers or oligonucleotide probes.[9,19–21] In one study, endomyocardial biopsy samples of 11 patients with idiopathic DCM or myocarditis were studied for the presence of enteroviral RNA using RT-NPCR; two were found positive, not only with enterovirus-specific primers but also with CVB-specific primers.[21] More recently, characterization was by restriction endonuclease digestion or, more definitively, by direct nucleotide sequencing of RT-PCR products and comparison with documented enterovirus sequences. Our study by the direct nucleotide sequencing of RT-NPCR products reveals that, in most cases, the sequences detected in the myocardial samples from patients with myocarditis or DCM show the greatest homology with group B Coxsackievirus, in particular, CVB3[22] (L. C. Archard and H. Zhang, unpublished). Kämmerer et al[23] reported that 10 of 47 endomyocardial biopsy samples from patients with DCM were positive for enteroviral RNA by RT-NPCR; restriction endonuclease analysis showed CVB3-specific patterns of cleavage products in five PCR products, whereas the genotype of the other 5 positive samples was unclear. In a more recent communication, these authors reported 14 of 58 (24.1%) endomyocardial biopsy samples from cases of DCM positive by RT-NPCR. Restriction endonuclease analysis or direct nucleotide sequencing identified the CVB3 genotype in eight samples, CVB2 in one, and CVB6 in one, and the remaining four samples were not identified.[24] Similarly, enteroviral RNA was detected by RT-NPCR in formalin-fixed, postmortem heart tissue from five of six patients with acute myocarditis[25]; direct nucleotide sequencing showed that four positive samples had greatest sequence homology with CVB3 and one with CVB5. Collectively, these data suggest a predominant etiological role of CVB3 in enteroviral myocarditis and DCM, indicating that the majority of cases of human enteroviral heart muscle disease may be prevented by immunization against only a few clinically important serotypes of enterovirus.

During investigations of enteroviral heart muscle disease in a mouse model, we derived several phenotypically distinguishable, attenuated variants of the cardiovirulent CVB3 strain Nancy, reactivated from a full-length cDNA clone by transfection of Vero cells.[26] These mutants are neutralized by CVB3-specific antisera. This report describes characterization of the stability of attenuated phenotype of these variants *in vivo* and their ability to elicit CVB3-specific neutralizing antibody and their prophylactic effect in a newly characterized murine model of CVB3-induced myocarditis.[27]

Materials and Methods

Virus

The prototypic cardiovirulent virus (rCVB3) was reactivated from a full-length, sequenced cDNA clone of CVB3 strain Nancy[28,29] by transfection of Vero cells. Serial dilutions of virus stocks were inoculated onto Vero cell monolayers to determine infectivity titer by plaque assay, as described previously.[27] The attenuated viruses p14V-1 (large plaque phenotype) and p14V-4 (small plaque phenotype) were derived from cardiovirulent rCVB3 by passage in cultured human dermatofibroblasts (HDF). Confluent HDF cells were inoculated with rCVB3 at low multiplicity of infection, 0.1 to 0.5 plaque-forming units (PFU)/cell, maintained in Dulbecco's modified minimal essential medium for 5 days and then subjected to freeze-thaw to release intracellular virus. Cell debris was pelletted by centrifugation and virus in the supernatant was titrated and used as inoculum for the next passage. This procedure was repeated (14 passages) until attenuated variants were identified by testing for cardiovirulence in mice.

Murine Models of Myocarditis

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication 85-23, revised 1985) and Animals (Scientific Procedures) Act 1986 UK. Five-week-old, male Balb/c (H-2^d) or SWR/Ola (H-2^q) mice were obtained from Harlan OLAC (Oxford, UK) and housed in a negative-pressure isolator after infection. The Balb/c mouse is an early model of CVB3-induced myocarditis with high mortality from multi-system disease, especially pancreatitis, whereas the SWR/Ola mouse strain has been characterized as a better model of CVB3 myocarditis with no mortality and only moderate pancreatitis.[27] To induce moderate to severe myocarditis, SWR/Ola mice were inoculated intraperitoneally (i.p.) with 10^3 or 10^5 PFU of rCVB3 or a cardiovirulent chimeric CVB3.[30] The former was used in this study. After sacrifice 7 days after infection, the heart was removed for histological and virological studies: one-third (apex) was fixed in 10%

formalin/PBS and the remainder was snap-frozen in liquid nitrogen and stored at −70°C.

Immunization with Attenuated Virus

Five-week-old, male SWR/Ola mice were distributed randomly into six groups of 10 mice each (except for group 6, which contained 5 mice). Groups 1, 2, and 3 were, respectively, inoculated with 10^3 PFU of cardiovirulent virus rCVB3, attenuant p14V-1, or p14V-4. Groups 4 and 5 were immunized by a single i.p. inoculation with 10^3 PFU of P14V-1 or P14V-4, respectively, and challenged with 10^3 or 10^5 PFU of rCVB3 14 days later. Group 6 received diluent (cell culture medium) only. Mouse blood was collected from the tail on days 0, 7, and 14 (groups 4 and 5 only) for specific antibody assays. Mice were sacrificed 7 days after inoculation (groups 1, 2, 3, and 6) or challenge (groups 4 and 5).

Histology

Formalin-fixed, paraffin-embedded sections (4 μm) were prepared and stained with hematoxylin and eosin (H&E) by standard procedures. Because of the focal nature of CVB3-induced myocarditis, four to six sections from each tissue sample at 40-μm intervals were examined by two examiners. The number of myocarditic lesions per section was counted microscopically and the mean score determined.[27,31]

Virus-Specific Antibody Assays

Neutralizing antibody titer was determined against 100 $TCID_{50}$ of prototypic rCVB3. Twofold dilutions of mouse serum were incubated with the virus before inoculation of Vero cell monolayers in 96-well microtiter plates. The cells were examined for cytopathic effect on days 4 and 7. The neutralizing antibody titer is expressed as the highest dilution of serum preventing the cytopathic effect in cell culture.

An indirect immunofluorescent (IF) technique was also used to detect virus-specific antibody. Mouse sera were incubated with rCVB3-infected Vero cells. After washing and incubation with a fluorescein-conjugated rabbit anti-mouse Ig conjugate (Dako, High Wycombe, UK), cells were examined by fluorescence microscopy. The IF antibody titer is expressed as the highest dilution of serum where virus-specific IF signals were observed.

In Vivo *Passage of Attenuant P14V-1*

The stability of attenuant p14V-1 was assessed by sequential passage through myocardium of SWR/Ola mice with occasional passage in Vero cells to increase its infectivity titer before further passage *in vivo*. Plaque-purified p14V-1 was passaged twice in Vero cells to prepare a virus stock and mice were inoculated i.p. with 10^6 to 10^7 PFU of this stock. The mice were sacrificed on day 5 and the hearts removed. Two-thirds of the myocardial tissue was snap-frozen in liquid nitrogen and homogenized in 1.5 ml of PBS containing 1% fetal calf serum and 50 μg/ml kanamycin using a Griffiths homogenizer. Virus was harvested from the homogenate as described for infected cells and titrated. A 0.5-ml aliquot of the filter-sterilized (Millipore; 0.22 μm) virus suspension was then inoculated into additional mice and the process repeated.

Detection of Virus and Viral RNA

Isolation and titration of virus in heart homogenates or serum were carried out by virus plaque assay.[27] Total RNA was extracted from frozen myocardial samples by the acid guanidinium thiocyanate, phenol-chloroform method of Chomczynski and Sacchi.[32] A RT-PCR system amplifying the glucose 6-phosphate dehydrogenase (G-6-PD) was employed to verify RNA extraction.[33] Viral genomic RNA was detected using an enterovirus-specific RT-NPCR that amplifies a 510-nucleotide sequence in the 5′ nontranslated region of enterovirus.[34] Nucleotide sequences of the primer sets are, for the first-stage PCR, F16 (forward) 5′ TTA AAA CAG CCT GTG GGT TG 3′ and F5 (reverse) 5′ TCA CCG GAT GGC CAA TCC AA 3′ and for the nested PCR, F3 (forward) 5′ CTG GTA TCA CGG TAC CTT TG 3′ and F8 (reverse) 5′ AAA CAC GGA CAC CCA AAG TA 3′. The sensitivity of this technique was assessed by investigating myocardium from a CVB3-infected SWR/Ola mouse. Briefly, myocardial tissue from a mouse infected with rCVB3 was homogenized, and virus infectivity titers were determined by plaque assay in cultured Vero cells. Aliquots of this heart homogenate were diluted 10-fold in the homogenated heart of normal mice, instead of phosphate buffer or culture medium, so that each dilution contained the same amount of total cellular RNA. The RNA extraction and enterovirus RT-NPCR were performed as described above.

Detection of Anti-Heart Antibodies

Serum samples were collected from SWR/Ola mice inoculated with 10^3 to 10^5 PFU of either attenuant p14V-1 or the cardiovirulent wild-type virus at day 0 (before inoculation), day 7, or day 25 after inocula-

Table 1. *Protection of SWR/Ola Mice by Attenuants p14V-1 or p14V-4*

Mouse group	n	Challenge dose (PFU)	Virus isolation	Myocarditis lesions (mean ± SEM)
1 (rCVB3)	10	NA	9/9*	9/9 (17.9 ± 3.2)
2 (p14V-1)	10	NA	10/10	6/10 (2.0 ± 0.4)
3 (p14V-4)	10	NA	10/10	7/10 (3.1 ± 0.9)
4 (p14V-1/rCVB3)	10	10^3 (8)	0/8	0/8 (0)
		10^5 (2)	0/2	0/2 (0)
5 (p14V-4/rCVB3)	10	10^3 (6)	0/5*	0/5 (0)
		10^5 (4)	0/4	0/4 (0)
6 (Diluent)	5	NA	0/5	0/5 (0)

Groups 1 to 3 were inoculated with 10^3 PFU of cardiovirulent virus rCVB3 or attenuant p14V-1 or pP14V-4, respectively. Groups 4 and 5 were immunized with 10^3 PFU of p14V-1 or p14V-4, respectively, and challenged with 10^3 or 10^5 PFU of rCVB3 14 days later. Group 6 received diluent only. NA, not applicable.

*Inoculation failure occurred to one mouse in group 1 and one mouse in group 5.

tion. Myocardial lysates were prepared from mock-infected SWR/Ola mouse tissue as described previously.[35] Myocardial proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels with a 3% stacking gel and transferred to nitrocellulose membranes (Hybond C Super, Amersham International, Buckinghamshire, UK) by electroblotting. The immobilized proteins were probed with mouse serum collected from mice at various times after inoculation with wild-type or attenuated virus at a dilution of 1/200 and then with secondary rabbit anti-mouse horseradish peroxidase conjugate. The immunoreactivity of mouse serum with heart muscle antigens was visualized by chemiluminescence and exposed to Hyperfilm ECL (Amersham International) as reported previously.[36] Results were quantitated by scanning desitometry.

Statistics

The Wilcoxon rank sum test was used for the statistical significance of differences between unpaired samples. A P value of <0.05 was regarded as statistically significant.

Results

Assessment of Attenuated Phenotype

Both the Balb/c and SWR/Ola mouse models of enterovirus-induced myocarditis were used to evaluate the phenotype of two attenuated variants, p14V-1 and p14V-4. Eighteen (group A) and 20 (group B) Balb/c mice were respectively inoculated i.p. with 10^5 PFU of either reactivated wild-type virus (rCVB3) or p14V-1, and their mortality was compared. Mice in group A lost body weight rapidly and began to die by day 5 after infection. By day 7, 17 of 18 (94.4%) mice in group A died of fulminating infection. In contrast, all the mice in group B inoculated with p14V-1 maintained or gained body weight and none died after infection. In the SWR/Ola model where mortality after CVB3 infection is not normally observed, inoculation with either p14V-1 or p14V-4 resulted in no or only a few small myocarditic lesions compared with severe myocarditis caused by infection with cardiovirulent rCVB3 (Table 1). In separate experiments, a 10^4-fold reduction in cardiovirulence of these attenuants was observed in each model.[26,27]

Stability of the Attenuated Phenotype of P14V-1

The attenuant p14V-1 was passaged sequentially 20 times *in vivo* in the myocardium of SWR/Ola mice. The infectivity titer of virus in the heart, titrated after each passage, was between $\log_{10}$ 3 and $\log_{10}$ 4.5 PFU/100 mg tissue. Viruses P8, -11, -14, -16, and -18, passaged *in vivo*, were additionally passaged once in Vero cells to prepare a higher infectivity titer ($\log_{10}$ 6.5 to $\log_{10}$ 7 PFU/0.5 ml dose). The high virus dose did not increase the infectivity titer in infected heart (data not shown). To determine the cardiovirulence of this passaged virus (P20), mice (four per group) were inoculated with 10^3 or 10^5 PFU of P20 and compared at day 7 with the mice infected similarly with rCVB3. The infectivity titer of P20 in myocardium was $\log_{10}$ 3.90 ± 0.14 PFU/100 mg for the 10^3 PFU group and $\log_{10}$ 4.13 ± 0.12 PFU/100 mg for the 10^5 PFU group, significantly lower than that of the cardiovirulent rCVB3 ($\log_{10}$ 5.47 ± 0.03 PFU/100 mg for the 10^3 PFU group, $P < 0.05$, and $\log_{10}$ 5.72 ± 0.27 PFU/100 mg for the 10^5 PFU group, $p < 0.05$). Histology showed that virus P20 induced only marginal myocarditis (Table 2) in SWR/Ola mice, comparable to the attenuant p14V-1 (Table 1),

Table 2. *Attenuated Phenotype of Virus P20 Compared with rCVB3*

Virus	Myocarditis lesions (mean ± SEM)	
	10^3 PFU/SWR mouse	10^5 PFU/SWR mouse
P20	2.5 ± 1.2 ($P < 0.001$)*	7.4 ± 1.6 ($P < 0.001$)*
rCVB3	19.8 ± 2.2	35.1 ± 3.2
p14V-1	2.0 ± 0.4†	ND

ND, not done.
*Significantly different from rCVB3.
†See Table 1.

whereas cardiovirulent virus rCVB3 caused severe myocarditis as expected (Table 2).

Detection of Virus-Specific Antibody

Before inoculation, sera from all the SWR/Ola mice used in this study were negative for CVB3-specific antibody as demonstrated by virus neutralization or IF at dilutions of 1:20 to 1:40 or 1:10, respectively. All sera collected 7 or 14 days after inoculation with CVB3 were positive for neutralizing antibody with two unexpected exceptions (Table 3). Neutralizing antibody titers varied in individuals from 1:20 to 1:640 although there was no significant difference in mean antibody titers between experimental groups. One mouse in group 1 did not develop myocarditis, apparently due to inoculation failure. One mouse in group 5, inoculated with p14V-4, did not have detectable virus-specific antibody when assayed on day 14, although specific antibody was generated as a result of subsequent challenge with cardiovirulent virus rCVB3; again, it seems that inoculation failure had occurred and so this mouse was not immunized. All sera positive for CVB3-specific neutralizing antibody were also positive by IF when tested using rCVB3-infected Vero cells as antigen. IF antibody titers ranged from 1:320 to >1:1280. There was no significant difference in neutralizing or IF antibody titer at 7 and 14 days after immunization (data not shown).

Protection of Mice by Immunization with Attenuants

As expected from previous studies of the SWR/Ola mouse model,[27] none of the SWR/Ola mice inoculated with either virulent or attenuated CVB3 died after infection. Mice were challenged with either 10^3 or 10^5 PFU of cardiovirulent virus rCVB3 14 days after immunization. The mice were sacrificed 7 days after challenge, and the heart and pancreas were examined histologically. None of 19 successfully immunized mice in groups 4 and 5 showed myocarditis or pancreatitis, evidence of efficient protection (Figure 1). Mice inoculated with the cardiovirulent rCVB3 only (group 1) developed typical myocarditis and pancreatitis (Figure 1), whereas mice inoculated with an attenuated variant (groups 2 and 3) developed no or marginal myocarditis with only a few, much smaller myocarditic lesions and mild pancreatitis. Mice injected with diluent only (group 6) had normal histology of the heart and pancreas. The one animal in group 5 that failed to generate virus-specific antibody after attempted immunization with attenuant p14V-4, developed myocarditis after challenge with 10^3 PFU of the cardiovirulent virus rCVB3. The mean myocarditic lesion score was 15.2 per section, comparable to that in unprotected mice inoculated with rCVB3 (group 1).

Isolation of Virus from Myocardium and Detection of Viral RNA

CVB3 was isolated at 7 days after infection from the hearts of all mice inoculated with either cardiovirulent virus rCVB3 or attenuated variants p14V-1 or p14V-4. The infectivity titer of rCVB3 in myocardium was significantly higher ($\log_{10}$ = 5.35 ± 0.14 PFU/100 mg) than either p14V-1 ($\log_{10}$ = 3.65 ± 0.19 PFU/100 mg; $P < 0.001$) or p14V-4 ($\log_{10}$ = 4.06 ± 0.14 PFU/100 mg; $P < 0.001$), but no significant difference was found between the titers of the two attenu-

Table 3. *Neutralizing Antibody Response to CVB3 in SWR/Ola Mice*

Mouse group	n	Before inoculation		After inoculation	
		Number positive	Titers	Number positive	Log_e titers (mean ± SEM)
1 (rCVB3)	10	0/10	<1:20–40	8/8*†	5.17 ± 0.16
2 (p14V-1)	10	0/10	<1:20–40	9/9†	4.99 ± 1.67
3 (p14V-4)	10	0/10	<1:20	10/10	5.15 ± 1.63
4 (p14V-1/rCVB3)	10	0/10	<1:20–40	10/10	4.57 ± 0.25
5 (p14V-4/rCVB3)	10	0/10	<1:20	9/9*	5.08 ± 0.33
6 (Diluent)	5	0/5	<1:20	0/5	<3.0

*Inoculation failure occurred in one mouse in group 1 and one mouse in group 5; both are excluded from this table.
†Insufficient serum from one mouse.

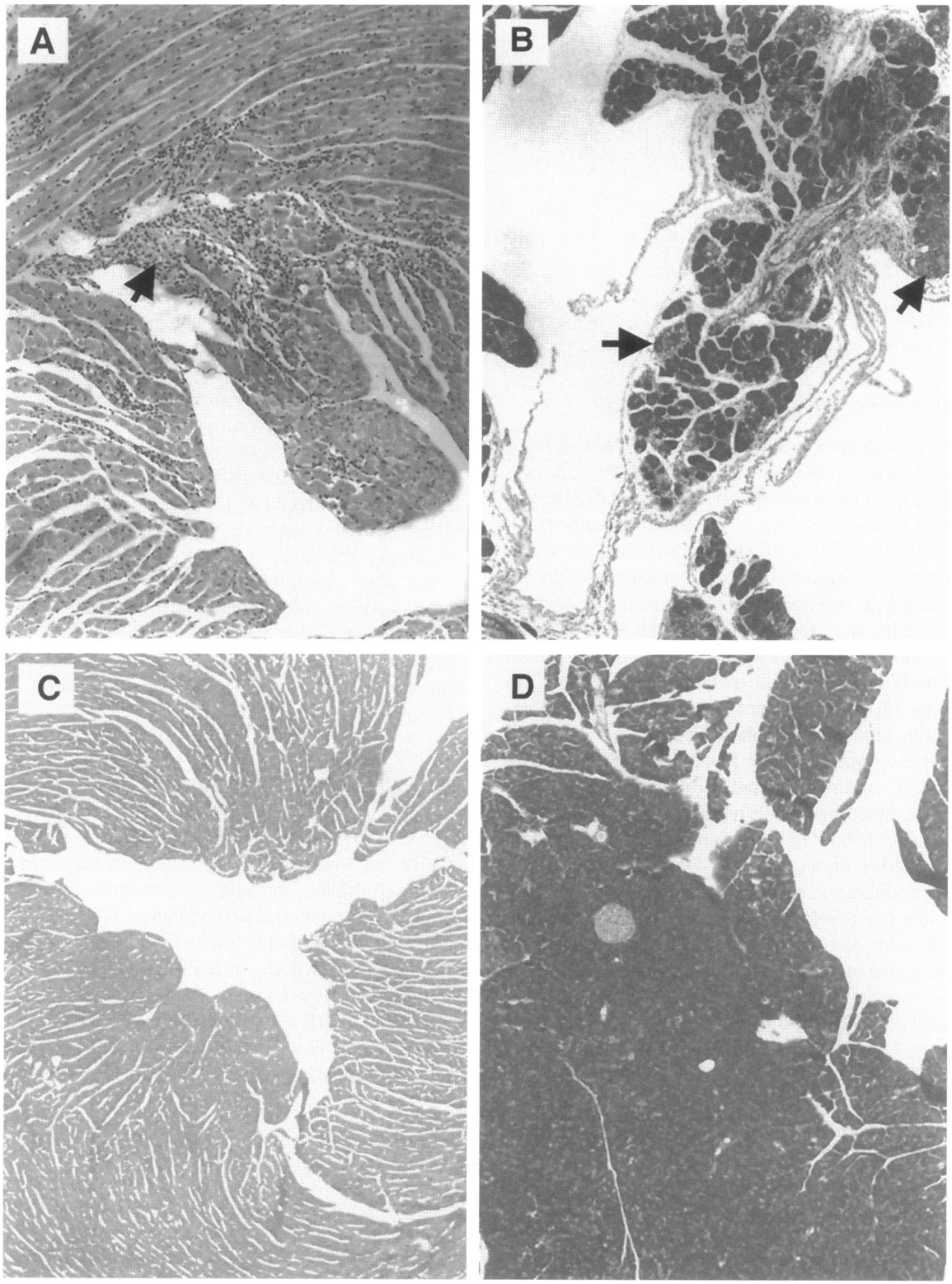

Figure 1. *Microphotographs of mouse heart and pancreas. Nonimmunized mice were sacrificed 7 days after inoculation with rCVB3. The 14-day immunized mice were sacrificed 7 days after subsequent challenge with rCVB3.* A *and* B: *Myocarditis (necrotic myofibers surrounded by inflammatory infiltrate;* (arrow) *or mild to moderate pancreatitis*[26] *(acinar necrosis* (arrows) *and disseminating infiltrate) from a mouse inoculated with cardiovirulent virus rCVB3.* C *and* D: *Normal heart or pancreas without apparent tissue necrosis and infiltration from a mouse protected by previous immunization with p14V-1. H&E; magnification, ×100* (A) *and ×50* (B *to* D).

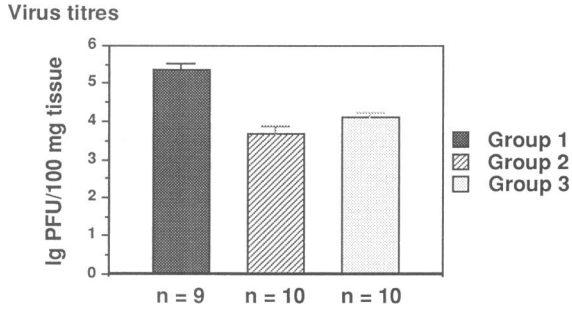

Figure 2. *Virus titers in mouse myocardium. Mice were sacrificed 7 days after inoculation with either rCVB3 (group 1), p14V-1 (group 2), or p14V-4 (group 3). Virus titer in heart homogenate was determined by plaque assay in Vero cells and expressed as $\log_{10}$ PFU per 100 mg of tissue. Significant differences were observed between group 1 and group 2 ($P < 0.001$) or group 1 and group 3 ($P < 0.01$).*

ants ($P > 0.05$; Figure 2). In contrast, virus was not isolated from either the myocardium or serum of an immunized mouse 7 days after challenge with cardiovirulent rCVB3.

Given that the plaque assay detecting infectious virus particles probably represents only 0.01 to 0.001 of the copies of virus genomic RNA present in infected cells, we employed the more sensitive technique of RT-NPCR to detect viral RNA in mouse myocardium. As the sensitivity of this technique was established previously by reconstitution of infected tissue,[34] it was retested in this study using 10-fold serial dilutions of an experimentally infected myocardial sample containing a known virus titer (PFU). Figure 3 shows that as few as 0.016 PFU of CVB3 (equivalent to 1.6 to 16 virus genomic RNA molecules, or 0.007 to 0.07 fg of virus genomic RNA) was detectable using RT-NPCR. Enteroviral RNA was detected in myocardium from all mice inoculated with either cardiovirulent or attenuated virus at 7 days after infection. In contrast, all mice immunized with p14V-1 or p14V-4 were negative for viral RNA in myocardium at 7 days after challenge with rCVB3 (Figure 4), demonstrating full protection by immunization and showing that the attenuants were no longer present in myocardium 21 days after inoculation. RNA extracts from all samples were positive for the G-6-PD coding sequence, confirming the integrity of the RNA templates.

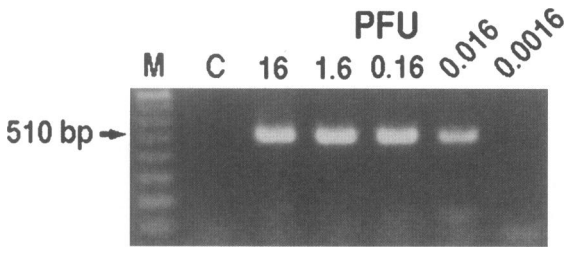

Figure 3. *Sensitivity of RT-NPCR. Total RNA was extracted from 10-fold dilutions of homogenated, CVB3-infected mouse myocardium with known virus infectivity and amplified using the RT-NPCR technique. The 510-bp NPCR products were visualized under ultraviolet light by ethidium bromide staining after electrophoresis in 1.5% agarose. As few as 0.016 PFU was detected (see text). M, 100-bp ladder markers (GIBCO-BRL); C, reagents and water control.*

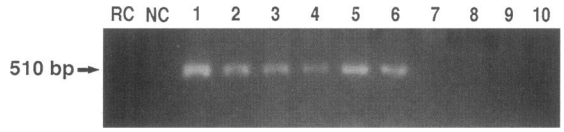

Figure 4. *Detection of enteroviral RNA from mouse myocardium by RT-NPCR. Total RNA was extracted from the samples collected 7 days after inoculation with rCVB3 (lanes 1 and 2), p14V-1 (lanes 3 and 4), or p14V-4 (lanes 5 and 6). Lanes 7 and 8 or 9 and 10, mice immunized with p14V-1 or p14V-4, respectively, 14 days before challenge with the wild type and killed 7 days after the challenge. The NPCR products were visualized under ultraviolet light by ethidium bromide staining after electrophoresis in 1.5% agarose. RC, PCR reagent and water control; NC, normal mouse control.*

Attenuant p14V-1 Induced a Transient Low Level of Anti-Cardiac Myosin Heavy Chain Antibody in SWR/Ola Mice

Serum samples from mock-infected, wild-type, or attenuant-infected mice were tested for anti-heart antibodies. Background signals of anti-myosin heavy chain reactivity was observed in all serum samples taken before virus inoculation (day 0) as well as in day 7 samples from the mock-infected mice (n = 3). A 7-fold increase in anti-myosin immunoreactivity, expressed in OD units, was seen in day 7 samples from mice (n = 4) inoculated with the cardiovirulent wild-type virus, whereas an increase of only 1.5-fold was seen in the samples from mice (n = 4) inoculated with the attenuant (Figure 5A). When serum samples collected from an independent experiment 25 days after infection were tested, an even bigger difference in the level of anti-myosin immunoreactivity was observed between mice inoculated with the wild-type and attenuated virus. The level of anti-myosin antibody remained high or increased yet further in four of five mice inoculated with the wild-type virus but, in contrast, had declined dramatically by this time in all five mice receiving the attenuant p14V-1 (Figure 5B).

Discussion

We derived attenuants p14V-1 and p14V-4 from a genetically defined cardiovirulent strain of CVB3 reactivated from a sequenced full-length cDNA clone by repeated passage in nonpermissive cells. CVB3-specific antibody neutralizes these attenuants,[26] demonstrating retention of neutralization epitopes of

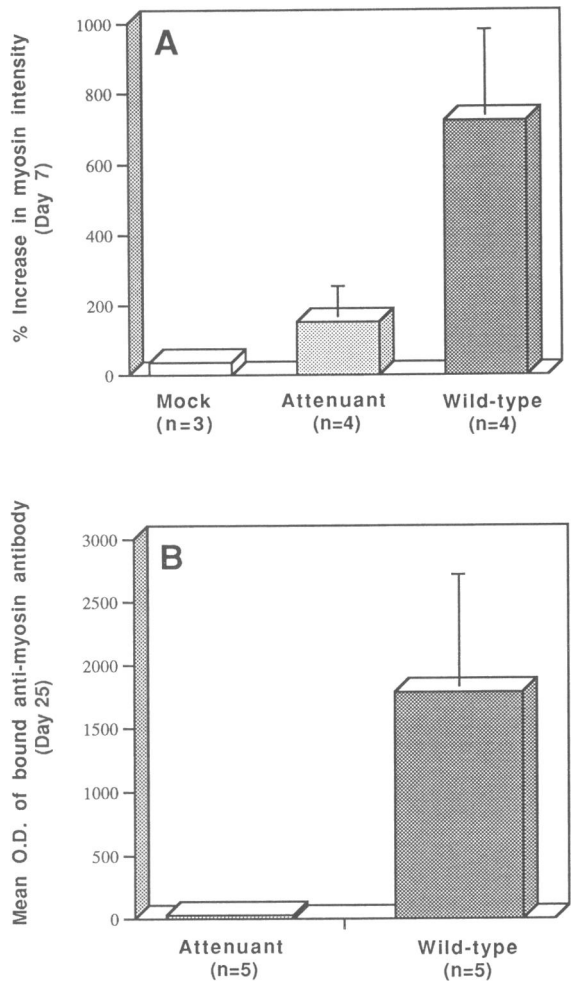

Figure 5. *Anti-myosin heavy chain antibody level in SWR mice after infection with attenuant or wild-type CVB3.* A: *Serum samples from mock, the attenuant p14V-1, or cardiovirulent wild-type virus-infected SWR/Ola mice were tested against the myocardial proteins of noninfected mice for anti-myosin immunoreactivity by Western blot. Percentage increase of autoantibody level was calculated from the OD units of anti-myosin antibody measured by densitometry on day 7 after infection compared with day 0 (before infection).* B: *Anti-myosin immunoreactivity 25 days after inoculation with either the attenuant p14V-1 or wild type.*

the wild-type virus. Although a specific mutation (80-Lys to Ser) has been identified at the amino terminus of the B-C loop of VP1 in p14V-1,[30] a region containing major neutralization epitopes as well as determinants of cellular immunity,[37–39] the data from this study suggest that this mutation does not alter neutralization epitopes. Attenuation of CVB has been achieved previously by other methods; for example, Trousdale et al[40] derived attenuated, temperature-sensitive mutants by propagating a cardiovirulent CVB3 at nonpermissive temperature. Although inoculation of neonatal CD-1 mice with temperature-sensitive mutant type 1 protected against CVB3 myocarditis during adolescence, anti-CVB3 neutralizing antibody was not detected in these inoculated mice and virus replication in the myocardium did occur,[41] making it unsuitable as a vaccine candidate. Prabhakar et al[42] derived CVB4 antibody-escape mutants with an attenuated phenotype by propagation of virus under selection pressure from CVB4-specific neutralizing monoclonal antibody; attenuated virus derived by this approach may have altered neutralization epitopes and, therefore, is unlikely to be useful for vaccine development.

In the present study, a low inoculum (10^3 PFU) of attenuant p14V-1 or p14V-4 elicited CVB3-specific neutralizing antibody in SWR/Ola mice within 1 week, with antibody titers similar to those elicited by infection with the cardiovirulent rCVB3. This antibody effectively neutralized cardiovirulent virus on subsequent challenge, before virus replication in the myocardium could be established. The mice were fully protected from development of myocarditis by these specific neutralizing antibodies as well as other mechanisms, eg, local production of interferon and cellular immunity, even when challenged with a 100-fold greater inoculum (10^5 PFU) of cardiovirulent wild-type virus. One mouse in an immunized group did not produce specific antibody due to inoculation failure; as a result, infectious virus and viral RNA were detected in myocardium, and typical focal myocarditis and pancreatitis were seen after challenge with the cardiovirulent wild type. Specific antibody was detected 7 days after the challenge. This random event complements the data obtained from protected mice.

The efficacy of live vaccines against enterovirus infection is demonstrated by the widespread use of attenuated Sabin polio vaccine.[43] A simple immunization course with a small inoculum of Sabin oral polio vaccine types 1 to 3 without adjuvant induces high-titer neutralizing antibody against all three poliovirus serotypes and long-lived immunity. After oral administration, the vaccine strains are propagated by enteric infection in vaccines and spread in the community by the natural route of fecal-oral infection. They also induce production of specific mucosal IgA, conferring local immunity that is important in the protection against enterovirus infection.[44] However, a major concern in the use of live, attenuated enterovirus vaccines arises from the observation that Sabin polio vaccine, particularly type 3, reverts to neurovirulence *in vivo* within 72 hours of administration and can cause vaccine-associated poliomyelitis.[45] In the present study, one CVB3 attenuant was phenotypically stable, at least *in vitro* or in the experimental animals; p14V-1 did not revert to the parental plaque or cardiovirulence phenotype after either 10 serial

passages in cultured Vero cells (data not shown) or 20 passages in SWR/Ola mouse heart *in vivo*.

Analogous to many reports that enteroviral RNA persists in the heart of patients with myocarditis or DCM when no infectious virus can be detected, studies on animal models of CVB infection demonstrate that virus may persist in myocardium beyond the initial inflammatory phase.[20,34,46,47] Consequently, we considered the possibility that the use of attenuated CVB3 as a live vaccine results in persistent infection, as genomic RNA of the cardiovirulent wild-type rCVB3 was detected in the myocardium of SWR/Ola mice up to 30 or 60 days after infection[34] (H. Zhang and L. C. Archard, unpublished). In the current study, we showed that the attenuants p14V-1 and p14V-4 did not persist in mouse myocardium as viral RNA was not detected at 21 days after immunization by the highly sensitive RT-NPCR capable of detecting approximately 0.02 PFU, a sensitivity similar to what we reported previously.[34] The possibility of virus persistence in other tissues is also considered and being investigated.

It is well documented that CVB3 infection can induce autoantibody reactive to cardiac myosin heavy chain in certain mouse strains, which is involved in the immunopathogenesis of viral myocarditis.[48] Anti-heart antibodies are also found in patients with myocarditis or DCM, and they are implicated in the pathogenesis of these heart muscle diseases.[36,49] We were concerned that immunization with the attenuants might induce autoimmunity to heart tissue. Results from this study show that, in contrast to the cardiovirulent wild-type virus, which induces high autoimmune reactivity to myosin heavy chain and which remains high after inflammation, attenuant p14V-1 induces transient, low-level anti-myosin antibody. This suggests that immunization with attenuant p14V-1 does not induce significant antoimmune heart muscle disease.

We note that these attenuants, although 10^4-fold less cardiovirulent, are not totally apathogenic and induce also some pancreatitis in mice, although this is substantially reduced compared with infection with the wild type. This effect may be disadvantageous in prophylactic immunization against heart muscle disease and it may be possible to derive additional nonpathogenic strains from these variants, based on nucleotide sequence comparison between wild-type and attenuated viruses.[30,50–53] This approach may lead also to the development of recombinant, multivalent live vaccines.[38,54]

Attenuants p14V-1 and p14V-4 prevent experimental murine myocarditis with high efficacy at least partly by the induction of CVB3-specific neutralizing antibody. Mutant p14V-1 is phenotypically stable after multiple passage in mouse heart. It induced only a transient, low level of anti-myosin antibody compared with the wild type, and neither of the attenuants persist in myocardium of SWR/Ola mice. This study indicates that development of CVB vaccines is feasible and that immunization may prevent human myocarditis and DCM caused by CVB infection.

References

1. Melnick JL: Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. Virology, ed 2. Edited by BN Field, DM Knipe. New York, Raven Press, 1990, pp 507–548
2. Woodruff JF: Viral myocarditis. Am J Pathol 1980, 101: 425–483
3. Cambridge G, MacArthur CGC, Waterson AP, Goodwin JF, Oakley CM: Antibodies to coxsackie B viruses in primary congestive cardiomyopathy. Br Heart J 1979, 41:13–23
4. Bowles NE, Richardson PJ, Olsen EJ, Archard LC: Detection of Coxsackie B virus specific sequences in myocardial biopsies from cases of myocarditis and dilated cardiomyopathy. Lancet 1986, i:1120–1122
5. Kandolf R: The impact of recombinant DNA technology on the study of enterovirus heart disease. Coxsackieviruses-A General Update. Edited by BM Friedman. New York, Plenum Press, 1988, pp 293–318
6. Tracy S, Wiegand V, McManus BM, Gauntt CJ, Pallansch MA, Beck MA, Chapman NM: Molecular approaches to enteroviral diagnosis in idiopathic dilated cardiomyopathy and myocarditis. J Am Coll Cardiol 1990, 15:1687–1693
7. Jin O, Sole MJ, Butany JW, Chia WK, McLaughlin PR, Liu P, Liew CC: Detection of enterovirus RNA in myocardial biopsies from patients with myocarditis and cardiomyopathy using gene amplification by polymerase chain reaction. Circulation 1990, 82:8–16
8. Weiss LM, Movahed LA, Billingham ME, Leary ML: Detection of coxsackievirus B3 RNA in myocardial tissues by the polymerase chain reaction. Am J Pathol 1991, 138:497–503
9. Schwaiger A, Umlauft F, Weyrer K, Larcher C, Lyons J, Muhlberger V, Dietze O, Grunewald K: Detection of enteroviral ribonucleic acid in myocardial biopsies from patients with idiopathic dilated cardiomyopathy by polymerase chain reaction. Am Heart J 1993, 126: 406–10
10. Andreoletti L, Hober D, Decoene C, Copin MC, Lobert PE, Dewilde A, Stankowiac C, Wattre P: Detection of enteroviral RNA by polymerase chain reaction in endomyocardial tissue of patients with chronic cardiac diseases. J Med Virol 1996, 48:53–59
11. Martino TA, Liu P, Sole MJ: Viral infection and the pathogenesis of dilated cardiomyopathy. Circ Res 1994, 74:182–188

12. Bowles NE, Why H, Richardson P, Archard LC: End-stage dilated cardiomyopathy, persistence of enterovirus RNA in myocardium at cardiac transplantation and lack of immune response. Circulation 1988, 80:1128–1136
13. Archard LC, Bowles NE, Cunningham L, Freeke CA, Olsen EGJ, Rose ML, Meany B, Why HJF, Richardson PJ: Molecular probes for detection of persisting enterovirus infection of human heart and their prognostic value. Eur Heart J 1991, 12(Suppl D):56–59
14. Why HJF, Richardson P, Archard LC: Clinical and prognostic significance of detection of enteroviral RNA in the myocardium of patients with myocarditis or dilated cardiomyopathy. Circulation 1994, 89:2582–2589
15. Bowles NE, Dubowitz V, Sewry CA, Archard LC: Dermatomyositis, polymyositis, and Coxsackie-B-virus infection. Lancet 1987, i:1004–1007
16. Yousef GE, Bell EJ, Mann GF, Murugesan V, Smith DG, McCartney RA, Mowbray JF: Chronic enterovirus infection in patients with postviral fatigue syndrome. Lancet 1988, i:146–150
17. Yoon J-W, Austin M, Onodera T, Notkins AL: Virus-induced diabetes mellitus: isolation of a virus from the pancreas of a child with diabetic ketoacidosis. N Engl J Med 1979, 300:1173–1179
18. King M, Shaikh A, Bidwell D, Voller A, Banatvala YE: Coxsackie-B-virus-specific IgM responses in children with insulin-dependent (juvenile-onset; type I) diabetes mellitus. Lancet 1983, i:1397–1399
19. Chapman NM, Tracy S, Gauntt CJ, Fortmueller U: Molecular detection and identification of enteroviruses using enzymatic amplification and nucleic acid hybridisation. J Clin Microbiol 1990, 28:843–850
20. Koide H, Kitaura Y, Deguchi H, Ukimura A, Kawamura K, Hirai K: Genomic detection of enteroviruses in the myocardium: studies on animal hearts with coxsackievirus B3 myocarditis and endomyocardial biopsies from patients with myocarditis and dilated cardiomyopathy. Jpn Circ J 1992, 56:1081–1093
21. Severini GM, Mestroni L, Falaschi A, Camerini F, Giacca M: Nested polymerase chain reaction for high-sensitivity detection of enteroviral RNA in biological samples. J Clin Microbiol 1993, 31:1345–1349
22. Khan M, Why H, Richardson P, Archard LC: Nucleotide sequencing of PCR products showes the presence of Coxsackie-B3 virus in endomyocardial biopsies from patients with myocarditis or dilated cardiomyopathy. Biochem Soc Trans 1994, 22:176S
23. Kämmerer U, Kunkel B, Korn K: Nested PCR for specific detection and rapid identification of human picornaviruses. J Clin Microbiol 1994, 32:285–291
24. Kämmerer U, Kunkel B, Bachmann K, Gunther, Korn K: Dilated cardiomyopathy and enteroviruses: a RT PCR study with direct sequencing shows mainly CBV3 in infected myocardium. International Society for Heart Research, XV: European Section Meeting, Copenhagen, June 8–11. Edited by S. Haunso, K. Kjeldsen. Bologna, Monduzzi Editore, 1994, pp 699–703
25. Nicholson F, Ajetunmobi JF, Li M, Shackleton EA, Starkey WG, Illavia SJ, Muir P, Banatvala JE: Molecular detection and serotypic analysis of enterovirus RNA in archival specimens from patients with acute myocarditis. Br Heart J 1995, 74:522–527
26. Zhang HY, Yousef GE, Cunningham L, Blake NW, Ouyang X, Bayston TA, Kandolf R, Archard LC: Attenuation of a reactivated myocarditic coxsackievirus B3, 5′-non-translated region does not contain major attenuation determinants. J Med Virol 1993, 41:129–137
27. Zhang HY, Yousef GE, Ouyang X, Archard LC: Characterisation of a murine model of myocarditis induced by a reactivated Coxsackievirus B3. Int J Exp Pathol 1994, 75:99–110
28. Kandolf R, Hofschneider PH: Molecular cloning of the genome of a cardiotropic Coxsackie B3 virus: full length reverse-transcribed recombinant cDNA generates infectious virus in mammalian cells. Proc Natl Acad Sci USA 1995, 82:4818–4822
29. Klump WM, Bergmann I, Muller BC, Ameis D, Kandolf R: Complete nucleotide sequence of infectious coxsackievirus B3 cDNA: Two intitial 5′ uridine residues are regained during plus-strand RNA synthesis. J Virol 1990, 64:1573–1583
30. Zhang H, Blake NW, Ouyang X, Pandolfino YA, Morgan-Capner P, Archard LC: A Single amino acid substitution in the capsid protein VP1 of coxsackievirus B3 (CVB3) alters plaque phenotype in Vero cells but not cardiovirulence in a mouse model. Arch Virol 1995, 140:959–966
31. Gauntt CJ, Gomez PT, Duffey PS, Grant JA, Trent DW, Witherspoon SM: Characterization and myocarditic capabilities of Coxsackievirus B3 variants in selected mouse strains. J Virol 1984, 52:598–605
32. Chomczynski P, Sacchi N: Single-step RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987, 162:156–159
33. Boehm T, Spillantini MG, Sofroniew MV, Surani MA, Rabbitts TH: Developmentally regulated and tissue specific expression of mRNAs encoding the two alternative forms of LIM domain oncogene rhombotin: evidence for thymus expression. Oncogene 1991, 6:695–703
34. Ouyang X, Zhang H, Trevor AB, Archard LC: Detection of Coxsackievirus B3 RNA in mouse myocarditis by nested polymerase chain reaction. Clin Diagn Virol 1995 3:233–245
35. Dunn MJ, Rose ML, Latif N, Bradd S, Lovegrove C, Seymour C, Pomerance A, Yacoub MH: Demonstration by Western blotting of antiheart antibodies before and after cardiac transplantation. Transplantation 1991 51: 806–812
36. Latif N, Baker CS, Dunn MJ, Rose ML, Brady P, Yacoub MH: Frequency and specificity of antiheart antibodies in patients with dilated cardiomyopathy detected using SDS-PAGE and Western blotting. J Am Coll Cardiol 1993 22:1378–1384
37. Minor PD, Ferguson M, Evans DMA, Almond JW: Anti-

genic structures of polioviruses of type 1, 2, and 3. J Gen Virol 1986, 67:1283–1291
38. Reimann B-Y, Zell R, Kandolf R: Mapping of a neutralizing antigenic site of Coxsackievirus B4 by construction of an antigen chimera. J Virol 1991, 65:3475–3480
39. Kutubuddin M, Simons J, Chow M: Identification of T-helper epitopes in the VP1 capsid protein of poliovirus. J Virol 1992, 66:3042–3047
40. Trousdale MD, Paque RE, Gauntt CJ: Isolation of coxsackievirus B3 temperature-sensitive mutants and their assignment to complementation groups. Biochem Biophys Res Commun 1977, 76:368–375
41. Gauntt CJ, Paque RE, Trousdale MD, Angen RJ, Barr DT, Lipotich: Temperature-sensitive mutant of Coxsackievirus B3 establishes resistance in neonatal mice that protects them during adolescence against Coxsackie B3-induced myocarditis. Infect Immun 1983, 39:851–864
42. Prabhakar BS, Srinivasappa J, Ray U: Selection of coxsackievirus B4 variants with monoclonal antibodies results in attenuation. J Gen Virol 1987, 68:865–869
43. Sabin AB: Oral poliovirus vaccine: history of its development and use and current challenge to eliminate poliomyelitis from the world. J Infect Dis 1985, 151: 420–436
44. Bendinelli M, Conaldi PG, Matteucci D: Interactions with the immune system. Coxsackieviruses: A General Update. Edited by M Bendinelli, H Friedman. New York, Plenum Press, 1988, pp 81–102
45. Evans DMA, Dunn G, Minor PD, Schild GC, Cann AJ, Stanway G, Almond JW, Currey JW, Maizel JV: Increased neurovirulence associated with a single nucleotide change in a noncoding region of the Sabin type 3 poliovaccine genome. Nature 1985, 314:548–550
46. Klingel K, Hohenadl C, Canu A, Albrecht M, Seemann M, Mall G, Kandolf R: Ongoing enterovirus-induced myocarditis is associated with persistent heart muscle infection, quantitative analysis of virus replication, tissue damage, and inflammation. Proc Natl Acad Sci USA 1992, 89:314–318
47. Leparc I, Fuchs F, Kopecka H, Aymard M: Use of polymerase chain reaction with a murine model of picornavirus-induced myocarditis. J Clin Microbiol 1993, 31:2890–2894
48. Rose NR, Herskowitz A, Neumann DA, Neu N: Autoimmune myocarditis: a paradigm of post-infection autoimmune disease. Immunol Today 1988, 9:117–120
49. Neumann DA, Burek CL, Baughman KL, Rose NR, Herskowitz A: Circulating heart-reactive antibodies in patients with myocarditis or cardiomyopathy. J Am Coll Cardiol 1990, 16:839–846
50. Martin A, Wychowski C, Couderc T, Crainic R, Hogle J, Girard M: Engineering a poliovirus type 2 antigenic site on a type 1 capsid results in a chimaeric virus which is neurovirulent for mice. EMBO J 1988, 7:2839–2847
51. Westrop GD, Wareham KA, Evans DMA, Dunn G, Minor PD, Magrath DI, Taffs F, Marsden S, Skinner MA, Schild GC, Almond JW: Genetic basis of attenuation of the Sabin type 3 oral poliovirus vaccine. J Virol 1989, 63: 1338–1344
52. Tu Z, Chapman NM, Hufnagel G, Tracy S, Romero JR, Barry WH, Zhao L, Currey K, Shapiro B: The cardiovirulent phenotype of coxsackievirus B3 is determined at a single site in the genomic 5′ nontranslated region. J Virol 1995, 69:4607–4618
53. Ramsingh AI, Collins DN: A point mutation in the VP4 coding sequence of Coxsackievirus B4 influences virulence. J Virol 1995, 69:7278–7281
54. Evans DJ, McKeating J, Meredith JM, Burke KL, Katrak K, John A, Ferguson M, Minor PD, Weiss RA, Almond JW: An engineered poliovirus chimaera elicits broadly reactive HIV-1 neutralizing antibodies. Nature 1989, 339:385–388