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Inoculation of the attenuated Coxsackievirus B3 *Sabin3-like* strain induces a protection against virulent CVB3 Nancy and CVB4 E2 strains in Swiss mice by both oral and intraperitoneal routes

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Abstract: We have previously addressed the question of whether the attenuating mutations of domain V of the Poliovirus IRES were specific for a given genomic context or whether they could be extrapolated to a genomic related virus, the Coxsackievirus B3 (CVB3). Accordingly, we have described that *Sabin3-like* mutation (U^{473}→C) introduced in the CVB3 genome led to a defective mutant with a serious reduction in translation efficiency. In this study, we assessed the protection provided by the *Sabin3-like* mutant against CVB3 infection. For this purpose, we analyzed, *in vivo*, the *Sabin3-like* phenotype in Swiss mice inoculated with CVB3 and CVB4 E2 prototype strains either by oral or intraperitoneal (i.p) routes and explored the capacity of this mutant to act as a vaccine vector after the challenge. The *Sabin3-like* RNA was detected by semi-nested PCR in different organs: heart, pancreas and intestine at 10 days post-inoculation with both oral and i.p routes. Additionally, we did not observe any histological alterations in heart and intestine tissues. RNA was detected in the different organs of all mice immunized with the *Sabin3-like* strain and challenged with either CVB3 or CVB4 E2 by oral route at 7 days post-challenge. In contrast, no histological alteration of heart or pancreas tissues was observed after challenge with both wild-strains. Interestingly, the detection of viral RNA in heart, pancreas and intestine of mice immunized by i.p route was negative at 7 days post-challenge with CVB3 and CVB4 E2, and mice were protected from myocarditis and pancreatitis.

Key words: Coxsackievirus B3; immunization; challenge; mice; vaccine.

Abbreviations: CVB, Coxsackievirus B; FCS, fetal calf serum; i.p, intraperitoneal; IRES, internal ribosome entry site; MEM, Eagle's minimal essential medium; NTR, non-translated region; ORF, open reading frame; PBS, phosphate buffered saline; PV, Poliovirus; TCID, tissue culture infectious dose.

Introduction

The Coxsackievirus group B (CVB), which includes six serotypes (CVB1-CVB6), belongs to the family *Picornaviridae* and the genus *Enterovirus,* comprising coxsackieviruses A and B, echoviruses, and polioviruses. Among these, Coxsackievirus B3 (CVB3) is an important human pathogen that induces acute and chronic viral myocarditis in children and young adults. About 5–50% of myocarditis and its end stage, dilated cardiomyopathy, are attributable to CVB3 infection (Kawai 1999; Maisch et al. 2002). In addition to heart disease, CVBs occasionally cause chronic inflammatory diseases of the pancreas (insulin-dependent type 1 diabetes mellitus, idiopathic chronic pancreatitis) and central nervous system (Huber & Ramsingh 2004).

The CVB3 genome comprises a positive-stranded RNA that is 7,400 nucleotides long; the single open reading frame (ORF) encodes a 2,200 amino acid long polyprotein that is processed during translation by two viral proteases. The ORF is flanked by 5' and 3' non-translated regions (NTRs), which encode no proteins, but are highly conserved structured RNA sequences that are important for virus replication and translation (Jackson & Kaminski 1995; Bhattacharyya & Das 2005).

Although CVB3 induces severe human diseases, there is currently no vaccine or therapeutic reagent in clinical use. The first study to develop a CVB-specific vaccine produced a temperature-sensitive mutant virus (Godney et al. 1988). Since that time, several types of candidate vaccine against CVB3 have been developed. A subunit vaccine containing the capsid protein (Fohlman et al. 1990) and several DNA vaccines (Henke

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et al. 2001; Kim et al. 2005, 2009) have shown protective effects after lethal dose challenge. Several attenuated virus variants have also been developed to protect against CVB3-induced diseases (Landau et al. 1990; Knowlton et al. 1996; Zhang et al. 1997).

The efficacy of live vaccines against enterovirus infection is demonstrated by the widespread use of attenuated Sabin polio vaccine (Sabin 1985). After oral administration, the vaccine strains are propagated by enteric infection in vaccines and spread in the community by the natural route of faecal-oral infection. Most of the mouse studies carried out so far used the intraperitoneal (i.p) route of immunization and infection. In addition, it was reported that oral vaccination is safe and easily administered, making it particularly suitable for protecting against infectious agents intruding bodies via the mucosal surface (Mason et al. 2002), such as pathogens that cause enteric, respiratory transmitted disease (Kong et al. 2001).

Albert Sabin (1995) obtained attenuated strains of each poliovirus (PV) serotype, which were unable to productively infect and destroy neuronal cells. Among the various mutations existing between the virulent PV strains and their attenuated counterparts, several play a direct role in PV neurovirulence and were qualified as attenuating mutations (Malnou et al. 2002). Interestingly, in the vaccine strain of each PV serotype, an attenuating mutation was found in the 5' NTR, at a position very near to each other within stem-loop V of the internal ribosome entry site (IRES; nucleotides 472, 480, and 481, respectively, for PV Sabin 3, 1, and 2). Experimental data led to the conclusion that the Sabin 3 mutation was the major attenuating mutation, and was sufficient to induce the principal characteristics of attenuation (La Monica & Racaniello 1989; Svitkin et al. 1990).

In view of the high morbidity of newborns infected with coxsackieviruses B3 as well as the fact that these viruses are involved in several cases of viral myocarditis in patients of all ages, an attenuated vaccine would be of significant health benefit. Accordingly, Ben M'hadheb-Gharbi et al. (2006, 2007) constructed equivalent attenuating mutations into the genomic context of CVB3 strains and reported that the *Sabin3-like* mutation (U^{473}→C) led to a serious impairment of the translation efficiency (Souii et al. 2013), a reduction of the virus titer and a destabilization of the secondary structure of the IRES. They also demonstrated that the inoculation, by oral route, of Swiss mice within the *Sabin3-like* strain did not show any histological alterations in heart tissue when compared to hearts of mice infected with the wild-CVB3 strain (Souii et al. 2013).

Herein, and on the basis of these previous published data, we aimed to investigate whether the CVB3 *Sabin3-like* mutant could induce a protective immunity against virulent CVB3 and CVB4 E2 prototype strains in Swiss mice by both oral and i.p routes. A systematic comparison is presented between the oral and i.p immunization detecting mutant and wild-type RNAs by PCR in heart, pancreas, and intestine tissues. In parallel, a histopathological study was carried out in order to assess the effect of the *Sabin3-like* infection on the histology of heart, pancreas and intestine tissues. Interestingly, this mutant was able to protect mice after challenge with virulent CVB3 and CVB4 E2 strains by both oral and i.p. routes. Additionally, we have noticed that the *Sabin3-like* strain did not provide full protection after the challenge with CVB4 E2 by i.p route. Nevertheless, this effect was relatively reduced compared to the one observed after challenge within the wild-strain.

Material and methods

Viruses

The CVB3 Nancy prototype strain, the CVB4 E2 diabetogenic strain, isolated in 1979 from the pancreas of a child who died from diabetic ketoacidosis (Yoon et al. 1979) and the CVB3 *Sabin3-like* strain used as a "vaccine candidate", obtained by direct mutagenesis (Ben M'hadheb-Gharbi et al. 2006), were propaged in Vero cells (African green monkey kidney cells) in Eagle's minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Sigma), 1% L-glutamine, 50 µg/mL streptomycin, 50 UI/mL penicillin (Bio Whittaker), 1% nonessential amino acids (Gibco BRL) and 0.05% Fongizone (Amphotericin B, Apothecon). Supernatants were collected 3 days after inoculation, clarified at 2,000 × *g* for 10 min, divided into aliquots and stored at −80 °C. Virus titers in stocks were determined in Vero cells by limiting dilution assays for 50% tissue culture infectious doses ($TCID_{50}$) according to Reed & Muench (1938) protocol.

Murine infection models

Swiss Albinos female mice (3–4 weeks old) obtained from Pasteur Institute (Tunisia), were divided into six groups. Three groups were orally inoculated, with, respectively, 1.26×10^6 $TCID_{50}$/mL CVB3 Nancy, 1×10^7 $TCID_{50}$/mL CVB4 E2 and 1.11×10^6 $TCID_{50}$/mL CVB3 *Sabin3-like*. In parallel, a negative control (uninfected) group was inoculated with phosphate buffered saline (PBS). For the i.p infection, mice were divided similarly as described above for oral route and received the same viral doses.

Mice were treated according to general ethic rules and maintained under specific pathogen-free conditions with unlimited access to food and water. All animals were sacrificed 10 days after inoculation. Portions of heart, pancreas, and intestine tissues were removed, rinsed with PBS, snap-frozen in liquid nitrogen and stored at −80 °C for PCR analysis or fixed in 10% formaldehyde for histological studies.

Immunization with the attenuated virus and challenge

Five-week old female Swiss Albinos mice were distributed randomly into four groups of 4 mice each. Groups 1 and 2 were immunized by a single oral or i.p inoculation with the attenuated *Sabin3-like* strain (1.11×10^6 $TCID_{50}$/mL) at days 0 and challenged by either oral and i.p inoculation at days 16, with, respectively, CVB3 (1.26×10^6 $TCID_{50}$/mL) and CVB4 E2 (1×10^7 $TCID_{50}$/mL) prototype strains. Control native mice were inoculated with PBS (uninfected controls). All animals were sacrificed 8 days after the challenge. Portions of heart, pancreas, and intestine tissues were removed, rinsed with PBS, snap-frozen in liquid nitrogen and stored at −80 °C for PCR analysis or fixed in 10% formaldehyde for histological investigations.

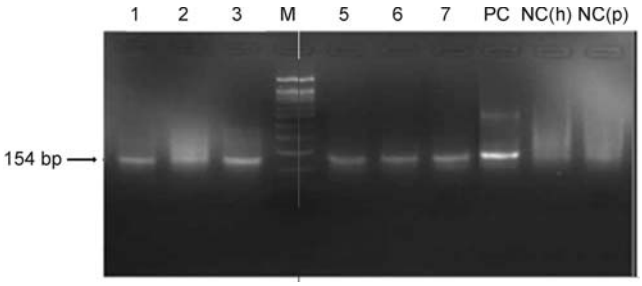

Fig. 1. Detection of enteroviral RNA from mice myocardium, pancreas and intestine by semi-nested PCR. Total RNA was extracted from samples collected from mice immunized orally with *Sabin3-like* 16 days before challenge with the wild type and sacrificed 7 days after the challenge by oral route. Lanes 1, 2 and 3: heart, pancreas and intestine, respectively, after challenge with CVB3 Nancy. Lanes 5, 6 and 7: heart, pancreas and intestine, respectively, after challenge with CVB4 E2. Lane M: 100 bp DNA ladder (Invitrogen). Lane PC: positive control (CVB3 Nancy reference strain). Lanes NC (h) and NC (p) correspond, respectively, to normal heart and normal pancreas controls. The semi-nested PCR products were visualized by ethidium bromide staining after electrophoresis on 2% agarose gel.

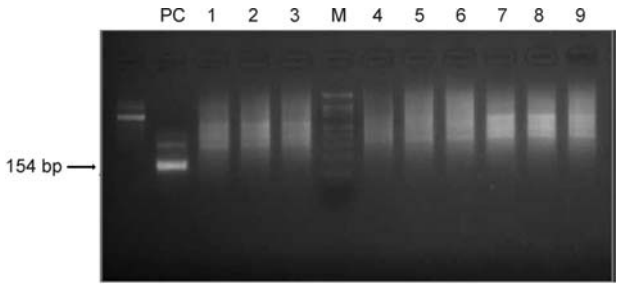

Fig. 2. Detection of enteroviral RNA in mice myocardium, pancreas and intestines by semi-nested PCR. Total RNA was extracted from mice organs immunized by i.p route with *Sabin3-like* 16 days before challenge with the wild strain and sacrificed 8 days after the challenge. Lanes 1, 2 and 3: hearts, pancreas and intestine, respectively, of normal control mice. Lanes 4, 5 and 6: heart, pancreas and intestine, respectively, after challenge with CVB3 Nancy. Lanes 7, 8 and 9: heart, pancreas and intestine, respectively, after challenge with CVB4 E2. Lane M: 100 bp DNA ladder (Invitrogen). Lane PC: positive control (CVB3 Nancy reference strain). Semi-nested PCR products were visualized by ethidium bromide staining after electrophoresis in 2% agarose gel.

Viral RNA detection

Viral RNA extraction. Total RNA was extracted from heart, pancreas and intestine of infected and uninfected mice using the acid guanidinium thiocyanate phenol-chloroform method (Chomczynski & Sacchi 1987). After ethanol precipitation, RNA was re-suspended in diethyl pyrocarbonate treated sterile water. Sterile PBS solution served as a negative control for the reaction.

Reverse transcription (RT). cDNA was synthesized in 50 µL volume containing 2 µg RNA, 10 mM Tris-HCl (pH 8.3), 75 mM KCl, 2.5 mM $MgCl_2$, 1 mM dNTP (Promega), 10 mM DiThioThreitol (DTT), 40 pmol antisense primer 007 (5'-ATTGTCACCATAAGCAGCCA3-3'), 1U RNase inhibitor (Amersham), 50 U Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) (Promega). The reaction was carried out at 42 °C for 30 min followed by a denaturation step during 5 min at 99 °C.

PCR amplification. PCR assay was performed using primers 006 (5'-TCCTCCGGCCCCTGAATGCG-3') and 007 (5'-ATTGTCACCATAAGCAGCCA-3') generating a 154 bp fragment. The amplification reaction was performed in a volume of 50 µL containing 100 ng cDNA, 25 mM Tris-HCl (pH 8), 50 mM KCl, 4 mM $MgCl_2$, 200 µM dNTP, 40 pmoles from each primer and 1U Taq DNA polymerase (Promega). Distilled water was included as a negative control. RNA extracted from Vero cells infected with CVB3 Nancy was reverse-transcribed, amplified according to the procedure already described above, and served as a positive control. Amplification was initiated by a denaturation step during 2 min at 95 °C followed by 30 cycles of 1 min denaturation at 95 °C, 1 min annealing at 42 °C and 1 min extension at 72 °C. Steps were followed by a final extension (10 min at 72 °C). All PCR products were electrophoresed on an agarose gel.

Semi-nested PCR. Amplification products showing negative results following the first amplification using primers (006/007) were used as a DNA template for a second amplification (semi-nested PCR) similar to the previous one except that the forward primer 006 was replaced by an external one called 005 (5'-CCAGCACTTCTGTTTCCCCGG-3'). This oligonucleotide is located within the 5' NTR, generating a 414 bp DNA fragment. Briefly, 40 ng of cDNA were added to 50 µL PCR mixture containing 40 µM of each primers (005/007), 25 mM Tris HCl (pH 8), 50 mM KCl, 4 mM $MgCl_2$, 200 µM dNTP and 1U of Taq DNA polymerase (Promega). Samples were denaturated for 2 min at 95 °C. Then, 30 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 45 °C, and extension for 1 min at 72 °C were performed. A final extension step for 7 min at 72 °C was also carried out. Semi-nested PCR products were analyzed by electrophoresis on an agarose gel containing 0.5 mg/mL ethidium bromide (Sigma) and visualized using the Gel Doc 2000 system (Bio-Rad).

Histology

Serial 3–5 µm thick sections of formalin-fixed and paraffin-embedded samples of heart, pancreas, and intestine tissues were stained with hematoxylin and eosin in a standard way and examined by light microscopy for the presence of inflammatory lesions, cellular infiltration, necrosis and muscle damage.

Results

Detection of viral RNA after immunization and challenge

CVB3 Nancy and CVB4 E2 RNAs were detected by semi-nested PCR in heart, intestine and pancreas tissues collected 10 days after the infection by oral and i.p routes within wild-CVB3 and wild-CVB4 E2 strains. The *Sabin3-like* RNA was detected in all organs collected 10 days post-inoculation with the attenuated CVB3 strain by oral and i.p routes (data not shown).

At 7 days post-challenge, RNA was detected in different organs of mice immunized with the*Sabin3-like* strain, and challenged with either CVB3 or CVB4 E2 by oral route (Fig. 1). In contrast, for all mice immunized by i.p route, RNA was not detected in any of the studied organs (Fig. 2). These results could be explicated by the fact that the viral RNA was no longer present in all organs 7 days after the challenge.

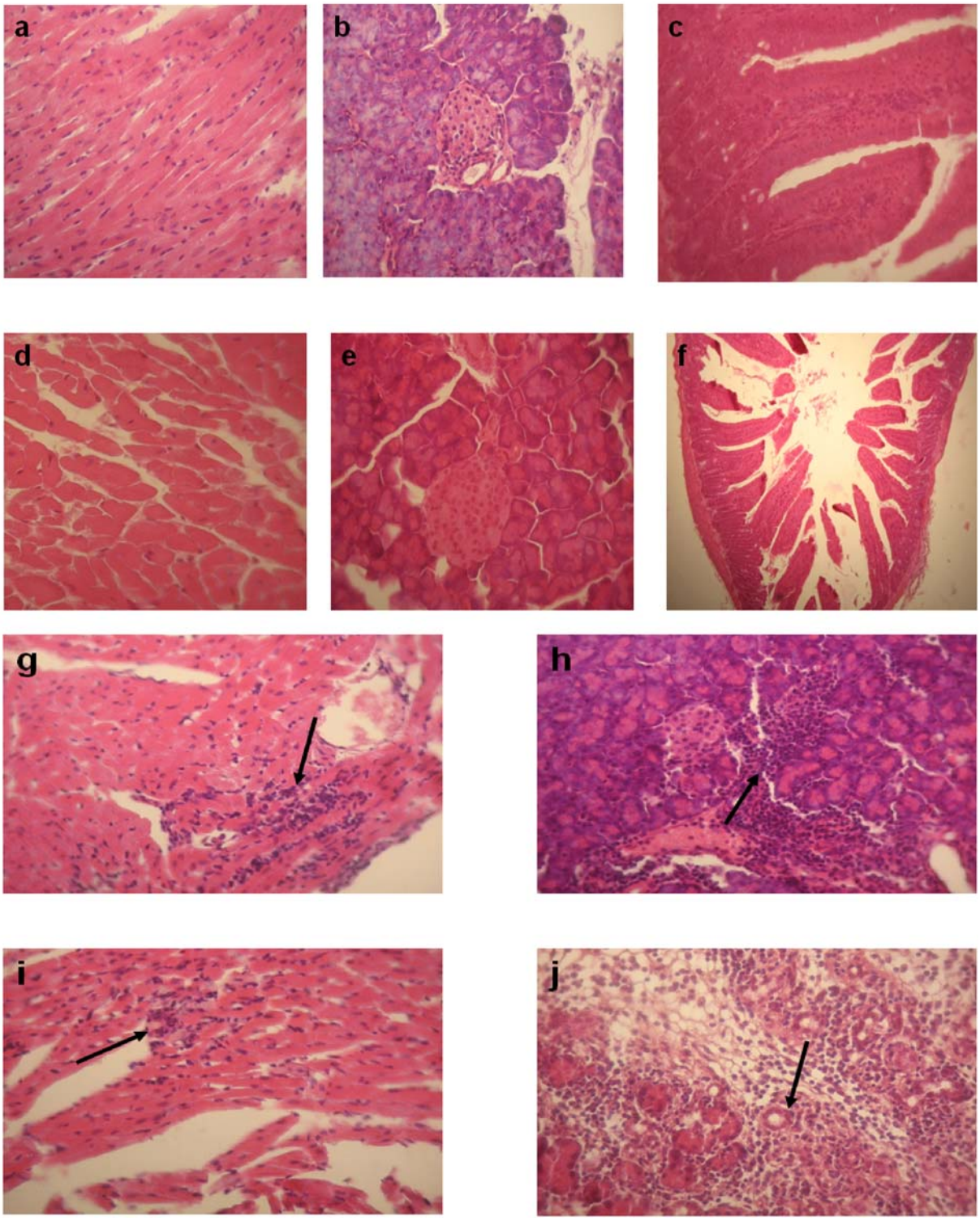

Fig. 3. Histology of the heart and pancreas of Swiss mice at day 10 after oral infection with mutant *Sabin3-like* or wild-type CVB3 and CVB4E2 viruses. Heart, pancreas and intestine were stained with hematoxylin and eosin. Shown are heart (a), pancreas (b) and intestine (c) from a control uninfected mouse, heart (d), pancreas (e) and intestine (f) from a mouse inoculated with CVB3*Sabin3-like*, heart (g) and pancreas (h) from a mouse inoculated with wild-type CVB3 and heart (i) and pancreas (j) from a mouse inoculated with CVB4 E2. Black arrow indicates inflammation area.

Mice immunization with the attenuated Sabin3-like strain

During all experiments, none of the Swiss Albinos mice inoculated with either virulent CVB3 Nancy or CVB4 E2 or the attenuated *Sabin3-like* strain died after oral or i.p infection. Hearts of mice inoculated with *Sabin3-like* by oral and i.p routes (Figs 3d, 4d) appeared normal, and were indistinguishable from the hearts of uninfected control mice (Figs 3a, 4a). However, a variable and a limited amount of pancreatic inflammatory disease was observed in some mice 10 days after *Sabin3-like* inoculation by i.p route (Fig. 4e). There was not any

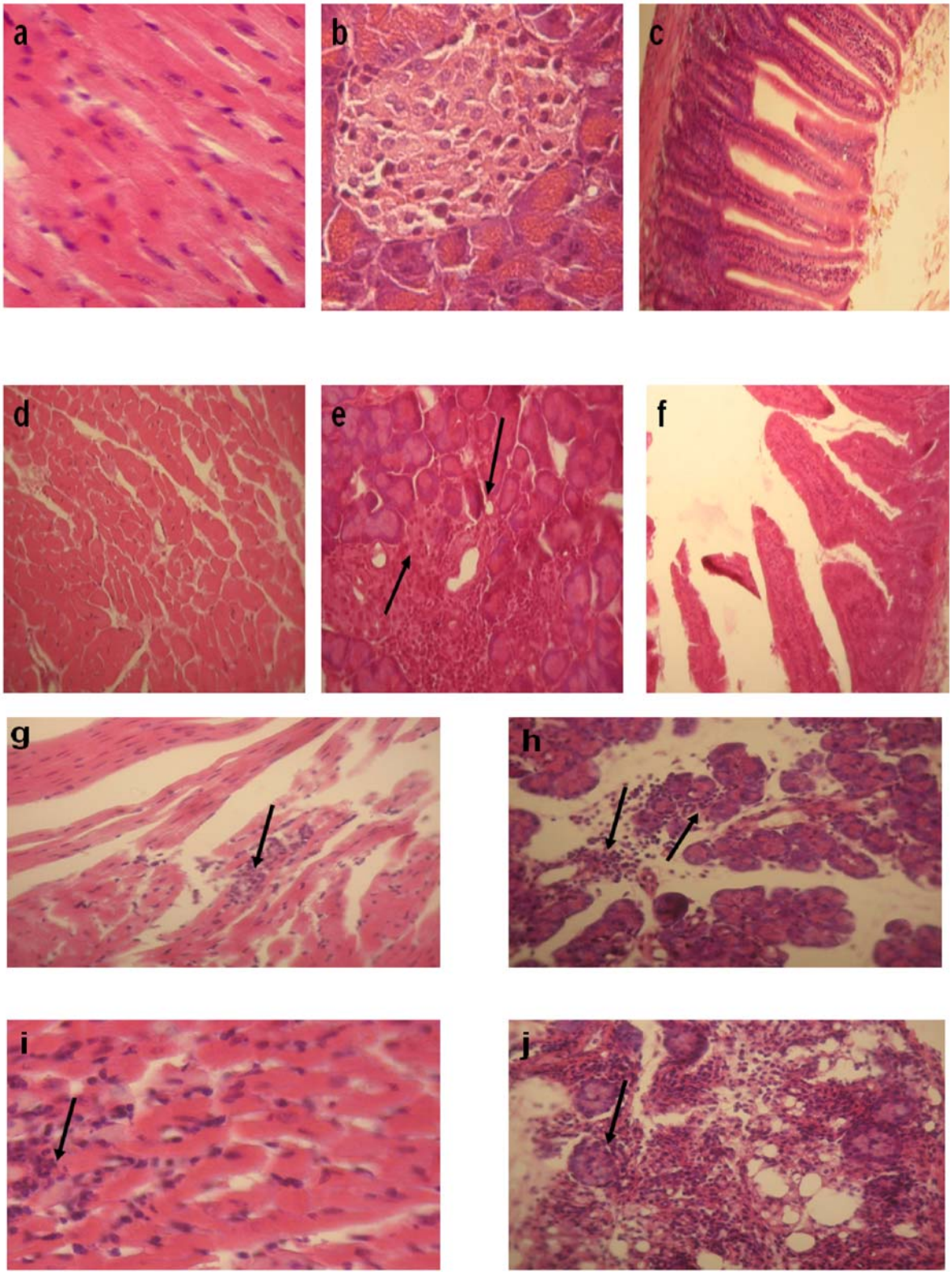

Fig. 4. Histology of the heart, pancreas and intestine of Swiss mice at day 10 after intraperitoneal infection with mutant *Sabin3-like* or wild-type CVB3 and CVB4 E2 strains. Heart, pancreas and intestine were stained with hematoxylin and eosin. Shown are heart (a), pancreas (b) and intestine (c) from a control uninfected mouse, heart (d), pancreas (e) and intestine (f) from a mouse inoculated with CVB3*Sabin3-like*, heart (g) and pancreas (h) from a mouse inoculated with wild-type CVB3 and heart (i) and pancreas (j) from a mouse inoculated with CVB4 E2. Black arrow indicates inflammation area.

evidence of pancreatic damage in mice inoculated by oral route (Fig. 3e), indistinguishable in pancreas from uninfected control mice (Fig. 3b).

In a marked contrast, both virulent CVB3 and CVB4 E2 strains induced inflammatory lesions with an extension infiltration areas and a necrosis in the heart muscle at 10 days post-infection by oral (Figs 3g–3i) and i.p (Figs 4g–4i) routes as well as a widespread in-

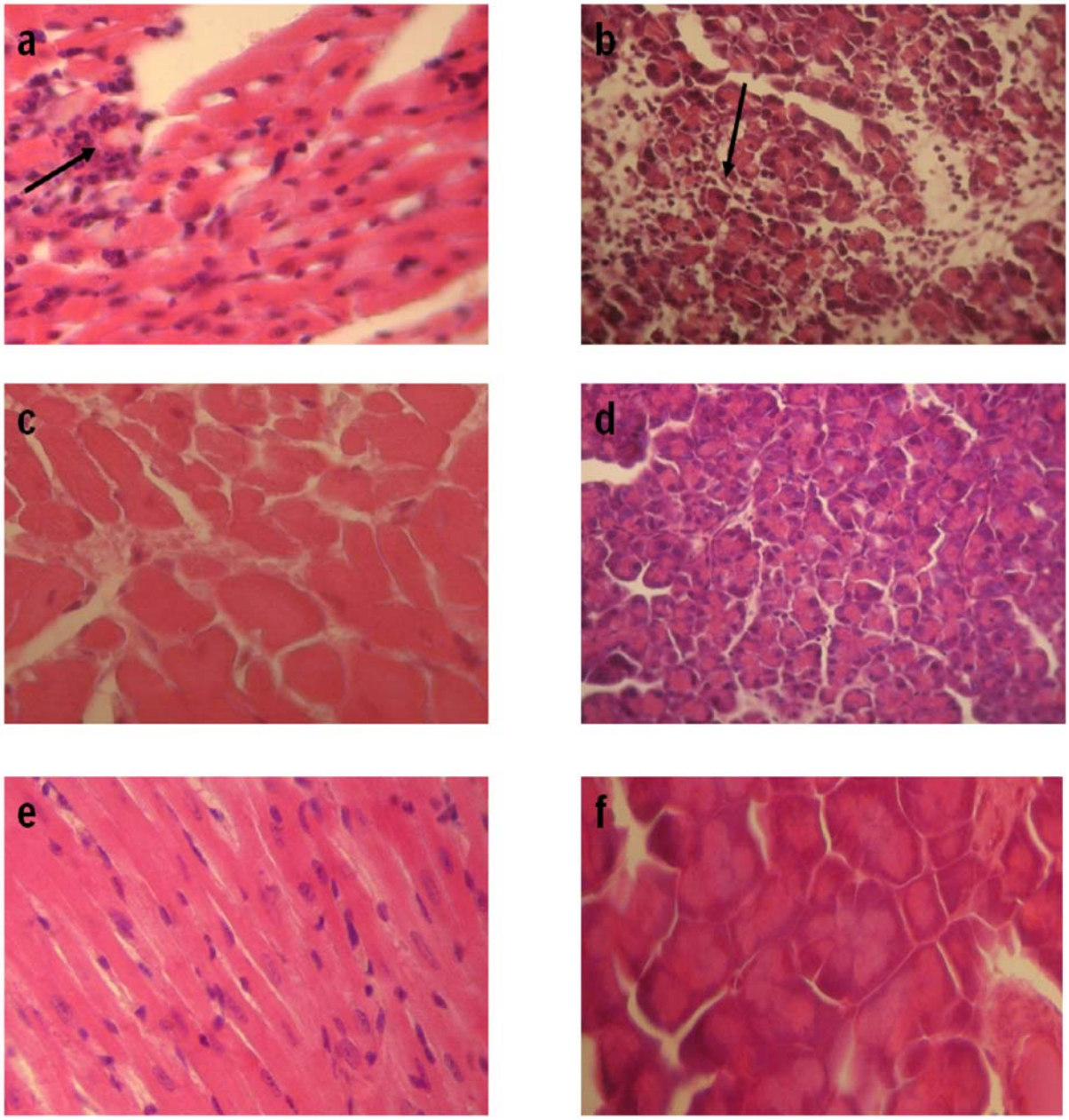

Fig. 5. Immunization of mice with CVB3 *Sabin3-like* by oral route. Mice were inoculated by oral route with non-supplemented medium (a, b) or CVB3 *Sabin3-like* (c, d, e, f). Mice were challenged 16 days later with either CVB3 Nancy (c, d) or CVB4E2 (e, f). Ten days after challenge, mice were sacrificed; hearts and pancreas were fixed in formalin, sectioned, and stained with hematoxylin and eosin. Shown are heart (a) (necrotic myofibres surrounded by inflammatory infiltrate) and pancreas (b) (mild to moderate pancreatitis with disseminating infiltrate) from a mouse inoculated with cardiovirulent virus CVB3 Nancy; and heart (c) and pancreas (d) from a mouse protected by previous immunization with CVB3 *Sabin3-like* and challenged with CVB3 Nancy. Heart (e) and pancreas (f) from a mouse protected by previous immunization with CVB3 *Sabin3-like* and challenged with CVB4 E2 are also shown. Black arrow indicates an inflammation area.

flammation in the acinar pancreatic tissue (Figs 3h–3j; Figs 4h–4j). The histological aspect of the intestine did not reveal any inflammation after both oral and i.p inoculations with CVB3 S*abin3-like* strain (Figs 3f–4f).

Protection of mice after challenge

In the present study, we assessed whether the CVB3 S*abin3-like* strain could induce a protective immunity against virulent CVB3 and CVB4 E2 infections. For this purpose, female Swiss Albinos mice were inoculated at day 0 with the attenuated *Sabin3-like* strain via either oral or i.p routes, and then challenged 16 days after, with, respectively, CVB3 Nancy and CVB4 E2 prototype strains. Control uninfected and unprotected mice were challenged at the same time. At 10 days post-challenge, the histology of heart, pancreas and intestine tissues was examined by light microscopy.

Control mice inoculated with PBS presented a normal histological aspect of the heart, pancreas and intestine. In contrast, control mice that had been challenged 16 days after inoculation with PBS with the CVB3 wild-strain showed a damage in heart and pan-

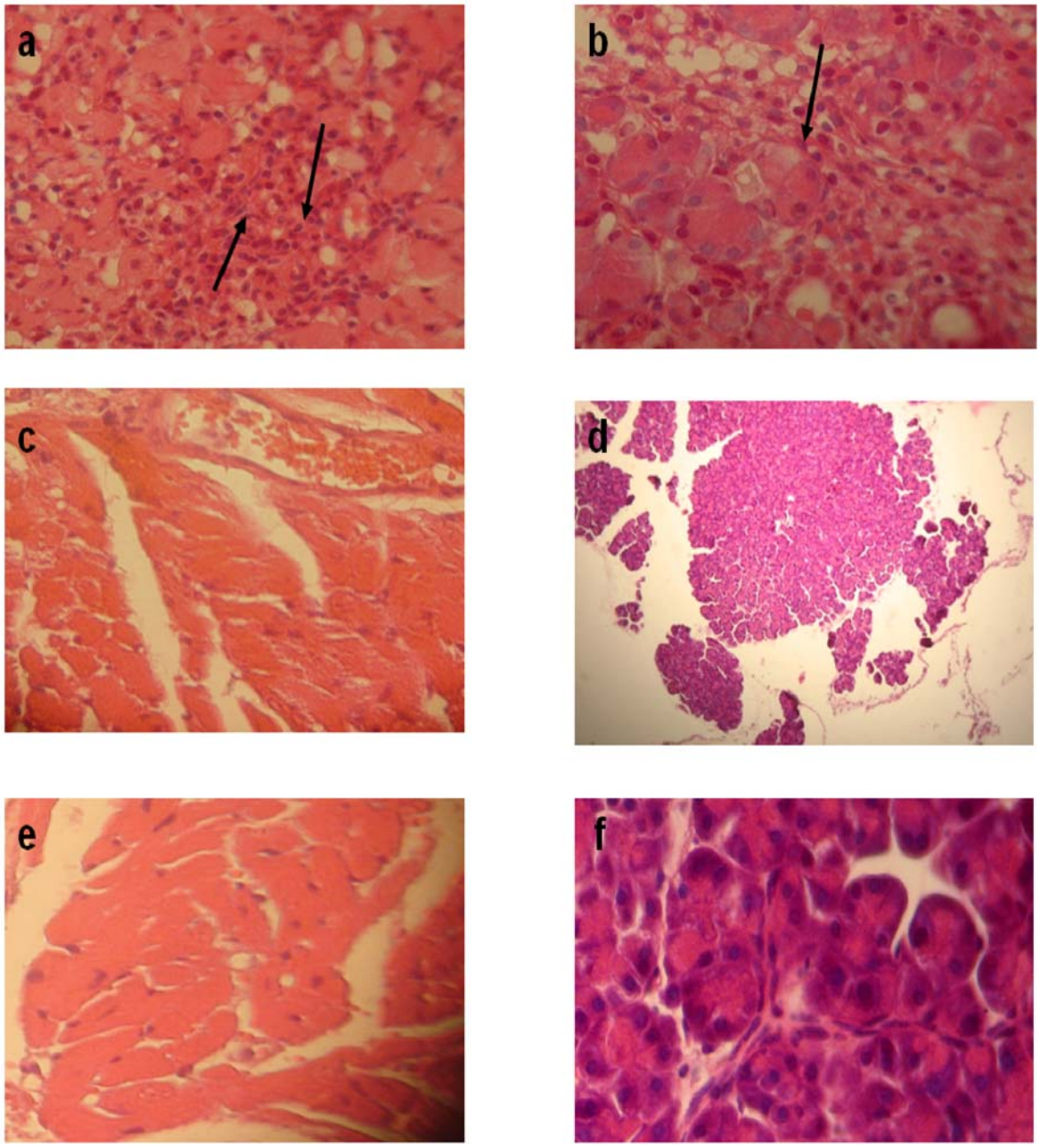

Fig. 6. Immunization of mice with CVB3 *Sabin3-like* by intraperitoneal route. Mice were inoculated by i.p route with non-supplemented medium (a, b) or CVB3*Sabin3-like* (c, d, e, f). Mice were challenged 16 days later with either CVB3 Nancy (c, d) or CVB4 E2 (e, f). Ten days after challenge, mice were sacrificed; hearts and pancreas were fixed in formalin, sectioned, and stained with hematoxylin and eosin. Shown are heart (a) and pancreas (b) from a mouse inoculated with cardiovirulent virus CVB3 Nancy. Heart (c) and pancreas (d) from a mouse protected by previous immunization with CVB3 *Sabin3-like* and challenged with CVB3 Nancy. Heart (e) and pancreas (f) from a mouse protected by previous immunization with CVB3*Sabin3-like* and challenged with CVB4 E2 are also shown. Black arrow indicates an inflammation area.

creas tissues (Figs 5a,b). Interestingly, mice challenged with CVB3 Nancy 10 days after inoculation by oral (Figs 5c,d) and i.p (Figs 6c,d) routes with the *Sabin3-like* strain did not show any histological alteration in hearts, pancreas and intestines. However, a little interstitial lymphocyte infiltration confined to the exocrine pancreas was observed in one mouse of three after challenge by oral route within virulent CVB3 Nancy. This finding was confirmed by PCR data.

Mice challenged with CVB4 E2, 10 days after inoculation by oral (Figs 5e,f) and i.p (Figs 6e,f) routes with CVB3 *Sabin3-like* did not show any inflammation or fibrosis pattern in heart, pancreas and intestine tissues. Although histology did not reveal any abnormalities after challenge by oral route, PCR results were positive for all organs. Nevertheless, one mouse immunized with the attenuated *Sabin3-like* strain, developed pancreatitis after challenge by i.p route with CVB4 E2.

Discussion

Coxsackievirus B3 is a principle etiologic agent in acute

and fulminant myocarditis (Tam 2006). Despite the well-characterized molecular structure of CVB3 (Bailey & Tapprich 2007) and well-demonstrated vaccination strategies in animal models (Henke et al. 2008), no efficient preventive vaccine against CVB3 is currently available. Herein, there is an obvious need to develop new and improved vaccines. In respect that CVB3 enters the host through the gastrointestinal tract; an ideal vaccine should be able to raise efficient protection at mucus surfaces to minimize initial infection and early viral dissemination.

Ben M'hadheb-Gharbi and collaborators (2006, 2007) have previously demonstrated that the *Sabin3-like* mutation introduced into the CVB3 genome affected the biological properties of the virus. In fact, this construct has shown a reduced replication in HeLa cell cultures and an impaired *in vitro* translation efficiency in standard rabbit reticulocyte lysates. They also reported that at 10 days post-inoculation with *Sabin3-like*, infected mice were protected from myocarditis.

In the present study, we demonstrate that the *Sabin3-like* strain did not induce any histological alterations in heart, pancreas and intestine tissues of infected mice; except some minor pancreatic inflammation in some mice at 10 days post-inoculation by i.p route. Importantly, this should not be surprising since our findings are in a perfect correlation with the data published by Slifka et al. (2001) and Lim et al. (2005) who reported that mice immunized with recombinant wild-type CVB3 suffered severe pancreatitis.

Inoculation of mice with the *Sabin3-like* strain by both oral and i.p routes protected mice from myocarditis. However, CVB3*Sabin3-like* RNA was detected in all mice organs collected 10 days post-infection by both oral and i.p routes. These results are also in agreement with the previously published data by Zhang et al. (1997), Chapman et al. (2000) and Dunn et al. (2000). Chapman et al. (2000) demonstrated that hearts of mice sacrificed at 5 days p.i., a time near the peak of cardiac virus titer, titers of both the chimeric (CPV/49) and the parental CVB3 strain were similar, averaging 2×10^5 $TCID_{50}$ units of virus/g of heart tissue. Therefore, the RNA of the mutant strain (CPV/49) was no detected in heart and pancreas on days 28 p.i.

Similarly to many reports that enteroviral RNA persists in the heart of patients with myocarditis 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 (Klingel et al. 1992; Leparc et al. 1993; Ouyang et al. 1995). Consequently, the use of attenuated CVB3 as a live vaccine results in persistent infection, as genomic RNA of the cardiovirulent wild-type CVB3 was detected in the myocardium and pancreas of mice up to 30 or 60 days after infection (Ouyang et al. 1995).

In the present work, we showed that the viral *Sabin3-like* RNA was detected by semi-nested PCR 10 days after inoculation by oral and i.p routes in all studied organs, but there was not any evidence of diseases (myocarditis and pancreatitis). Thus, it would be interesting to analyze the persistence of the attenuated RNA in heart and pancreas at 20 or 28 days post-immunization.

The mutant *Sabin3-like* construct proved to be protective when tested in mice. In fact, a single inoculation of the mutant *Sabin3-like* by i.p route protected all mice challenged 16 days later with a virulent CVB3 and CVB4 E2 which induced, respectively, myocarditis and pancreatitis in the unprotected control mice. Heart and pancreatic damages due to viral CVB3 and CVB4 replication have been demonstrated to be clearly evident at 10 days post-infection (Tracy et al. 1992; Tu et al. 1995; Bopegamage et al. 2003, 2005; Jaidane et al. 2006). However, one mouse immunized with the attenuated *Sabin3-like* strain, developed pancreatitis after challenge by i.p route with CVB4 E2. While the *Sabin3-like* strain replicates in mice but with a lower efficiency compared to the wild-CVB3 strain, it can induce either little or no myocarditis or pancreatitis of susceptible mice. Therefore, it would be interesting to see if the viral RNA can be detected at 20 days post-challenge in heart, pancreas and intestine.

In summary, the data presented here demonstrate that the *Sabin3-like* strain rapidly induced a protective immune response in the murine model and is sufficiently attenuated to act as an avirulent strain. Therefore, this construct could act as a promising protective vaccine against CVB3 and CVB4 E2 induced myocarditis and pancreatitis. This attenuated strain was not totally protective after challenge with CVB4 E2, although this effect is relatively reduced compared to the wild-type strain. Further investigations are needed to confirm these findings. Hence, the identification of humoural and cellular immune responses to the CVB3*Sabin3-like* strain, in conjunction with the analysis of neutralizing antibody induced by this strain is clearly a challenge in the near future to increase our knowledge of the coxsackievirus B3 cardiovirulence and attenuation.

References

Bailey J.M. & Tapprich W.E. 2007. Structure of the 5' nontranslated region of the coxsackievirus B3 genome: chemical modification and comparative sequence analysis. J. Virol. **81:** 650–668.

Ben M'hadheb-Gharbi M., Gharbi J., Paulous S., Brocard M., Komaromva A., Aouni M. & Kean K. 2006. Effects of the *Sabin-like* mutations in domain V of the internal ribosome entry segment on translational efficiency of Coxsackievirus B3. Mol. Genet. Genomics **276:** 402–412.

Ben M'hadheb-Gharbi M., Paulous S., Aouni M., Kean K.M. & Gharbi J. 2007. The substitution $U_{475} \rightarrow C$ with *Sabin3-like* mutation within the IRES attenuate Coxsackievirus B3 cardiovirulence. Mol. Biotechnol. **36:** 52–60.

Bhattacharyya S. & Das S. 2005. Mapping of secondary structure of the spacer region within the 5'-untranslated region of the coxsackievirus B3 RNA: possible role of an apical GAGA loop in binding La protein and influencing internal initiation of translation. Virus Res. **108:** 89–100.

Bopegamage S., Borsanyiova M., Vargova A., Petrovicova A., Benkovicova M. & Gomolcak P. 2003. Coxsackievirus infection of mice. I. Viral kinetics and histopathological changes in mice experimentally infected with coxsackieviruses B3 and B4 by oral route. Acta Virol. **47:** 245–251.

Bopegamage S., Kovacova J., Vargova A., Motusova J., Petrovicova A., Bencovicova M., Gomolacak P., Bakkers J., Van Kuppeveld F., Melchers W.J.G. & Galama J.M. 2005. Coxsackie B virus infection of mice: inoculation by oral route protects the pancreas from damage, but not from infection. J. Gen. Virol. **86:** 3271–3280.

Chapman N.M., Ragland A., Smith L.J., Höfling K., Willian S., Semler B. & Tracy S. 2000. A group B Coxsackievirus/poliovirus 5' nontranslated region chimera can act as an attenuated vaccine stain in mice. J. Virol. **74:** 4047–4056.

Chomczynski P. & Sacchi N. 1987. Single-step RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:** 156–159.

Dunn J.J., Chapman N.M., Tracy S. & Romero J.R. 2000. Genomic determinants of cardiovirulence in coxsackievirus B3 clinical isolates: localization to the 5' nontranslated region. J. Virol. **74:** 4787–4794.

Fohlman J., Ilbäck N.G., Friman G. & Morein B. 1990. Vaccination of Balb/c mice against enteroviral mediated myocarditis. Vaccine **8:** 381–384.

Godney E.K., Arizpe H.M. & Gaunti C.J. 1988. Characterization of the antibody response in vaccinated mice protected against Coxsackievirus B3-induced myocarditis. Viral Immunol. **1:** 305–314.

Henke A., Jarasch N., Martin U., Wegert J., Wildner A., Zell R. & Wutzler P. 2008. Recombinant coxsackievirus vectors for prevention and therapy of virus-induced heart disease. Int. J. Med. Microbiol. **298:** 127–134.

Henke A., Zell R. & Stelzner A. 2001. DNA vaccine-mediated immune responses in Coxsackievirus B3–infected mice. Antiviral Res. **49:** 49–54.

Huber S. & Ramsingh A.I. 2004. Coxsackievirus-induced pancreatitis. Viral Immunol. **17:** 358–369.

Jackson R.J. & Kaminski A. 1995. Internal initiation of translation in eukaryotes: the picornavirus paradigm and beyond. RNA **1:** 985–1000.

Jaidane H., Gharbi J., Lobert P.E., Lucas B., Hiar R., Ben M'hadheb M., Brilot F., Geenen V., Aouni M. & Hober D. 2006. Prolonged viral RNA detection in blood and lymphoid tissues from Coxsackievirus B4E2 orally-inoculated Swiss mice. Microbiol. Immunol. **50:** 971–974.

Kawai C. 1999. From myocarditis to cardiomyopathy: mechanisms of inflammation and cell death: learning from the past for the future. Circulation **99:** 1091–1100.

Kim D.S., Cho Y.J., Kim B.G., Lee S.H. & Nam J.H. 2009. Systematic analysis of attenuated Coxsackievirus expressing a foreign gene as a viral vaccine vector. Vaccine **28:** 1234–1240.

Kim J.Y., Jeon E.S., Lim B.K., Kim S.M., Chung S.K. & Kim J.M. 2005. Immunogenicity of a DNA vaccine for coxsackievirus B3 in mice: protective effects of capsid proteins against viral challenge. Vaccine **23:** 1672–1679.

Klingel K., Hohenadl C., Canu A., Albrecht M., Seemannn M., Mall G. & Kandolf R. 1992. 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 **89:** 314–318.

Knowlton K.U., Jeon E.S., Berkley N., Wessely R. & Huber S. 1996. A mutation in the puff region of VP2 attenuates the myocarditic phenotype of an infectious cDNA of the Woodruff variant of coxsackievirus B3. J. Virol. **70:** 7811–7818.

Kong Q., Richter L., Yang Y.F., Arntzen C.J., Mason H.S. & Thanavala Y. 2001. Oral immunization with hepatitis B surface antigen expressed in transgenic plants. Proc. Natl. Acad. Sci. USA **98:** 11539–11544.

La Monica N. & Racaniello V.R. 1989. Differences in replication of attenuated and neurovirulent polioviruses in human neuroblastoma cell line SH-SY5Y. J. Virol. **63:** 2357–2560.

Landau B.J., Whittier P.S., Finkelstein S.D., Alstein B., Grun J.B. & Schultz M. 1990. Induction of heterotypic virus resistance in adult inbred mice immunized with a variant of Coxsackievirus B3. Microb. Pathog. **8:** 289–298.

Leparc I., Fuchs F., Kopecka H. & Aymard M. 1993. Use of polymerase chain reaction with a murine model of picornavirus-induced myocarditis. J. Clin. Microbiol. **31:** 2890–2894.

Lim B.K., Shin J.O., Lee S.C., Kim D.K., Choi D.J. & Choe S.C. 2005. Long-term cardiac gene expression using a coxsackieviral vector. J. Mol. Cell. Cardiol. **38:** 745–751.

Maisch B., Ristic A.D., Hufnagel G. & Pankuweit S. 2002. Pathophysiology of viral myocarditis: the role of humoral immune response. Cardiovasc. Pathol. **11:** 112–122.

Malnou C.E., Pöyry T.A., Jackson R.J. & Kean K.M. 2002. Poliovirus internal ribosome entry segment structure alterations that specifically affect function in neuronal cells: molecular genetic analysis. J. Virol. **76:** 10617–10626.

Mason H.S., Warzecha H., Mor T. & Arntzen C.J. 2002. Edible plant vaccines: applications for prophylactic and therapeutic molecular medicine. Trends Mol. Med. **8:** 324–329.

Ouyang X., Zhang H., Trevor A.B. & Archard L.C. 1995. Detection of Coxsackievirus B3 RNA in mouse myocarditis by nested polymerase chain reaction. Clin. Diagn. Virol.**3:** 233–245.

Reed L.J. & Muench H. 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27:** 493–497.

Sabin A.B. 1985. Oral poliovirus vaccine: history of its development and use and current challenge to eliminate poliomyelitis from the world. J. Infect. Dis. **151:** 420–436.

Sabin A.B. 1995. Oral poliovirus vaccine. History of its development and prospects for eradication of poliomyelitis. JAMA **194:** 872–876.

Slifka M.K., Pagarigan R., Mena I., Feuer R. & Whitton J.L. 2001. Using recombinant coxsackievirus B3 to evaluate the induction and protective efficacy of CD8+ T cells during picornavirus infection. J. Virol. **75:** 2377–2387.

Souii A., Ben M'hadheb-Gharbi M., Sargueil B., Brossard A., Chamond N., Aouni M. & Gharbi J. 2013. Ribosomal initiation complex assembly within the wild-strain of Coxsackievirus B3 and live-attenuated *Sabin3-like* IRESes during the initiation of translation. Int. J. Mol. Sci. **14:** 4400–4418.

Svitkin Y.V., Cammack, N., Minor P.D. & Almon J.W. 1990. Translation deficiency of the Sabin type 3 poliovirus genome: association with an attenuating mutation C472-U. Virology **175:** 103–109.

Tam P.E. 2006. Coxsackievirus myocarditis: interplay between virus and host in the pathogenesis of heart disease. Viral Immunol. **19:** 133–146.

Tracy S., Chapman N.M. & Tu Z. 1992. Coxsackievirus B3 from an infectious cDNA copy of the genome is cardiovirulent in mice. Arch. Virol. **122:** 399–409.

Tu Z., Chapman N.M., Hufnagel G., Tracy S., Romero J.R., Barry W.H., Zhao L., Currey K. & Shapiro B. 1995. The cardiovirulent phenotype of coxsackievirus B3 is determined at a single site in the genomic 5' nontranslated region. J. Virol. **69:** 4607–4618.

Yoon J.W., Austin M., Onodera T. & Notkins A.L. 1979. Isolation of a virus from the pancreas of a child with diabetic ketoacidosis. New Engl. J. Med. **300:** 1173–1179.

Zhang H., Morgan-Capner P., Latif N., Pandolfino Y.A., Fan W. & Dunn M.J. 1997. Coxsackievirus B3-induced myocarditis. Characterization of stable attenuated variants that protect against infection with the cardiovirulent wild-type strain. Am. J. Pathol. **150:** 2197–2207.

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