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Short communication

DNA vaccine-mediated immune responses in Coxsackie virus B3-infected mice

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Abstract

DNA immunizations with the major structural protein VP1 of Coxsackie virus B3 (CVB3) have been previously found to protect BALB/c mice from lethal challenge. Here we report that the other CVB3 capsid proteins, VP2, VP3, and VP4, were less effective at preventing CVB3-caused disease. The application of pCMV/VP1 as a vaccine caused decreased myocyte destruction, reduced viral load in the heart tissue, accelerated antibody induction, and an early cytokine expression in heart tissue. In summary, our results indicate that the induction of B cell and/or T cell memory in vaccinated mice prior to challenge is responsible for the protection observed.

Keywords: DNA vaccine; Immune response; Coxsackie virus

Infections with Coxsackie virus B3 (CVB3) are common causes of acute and chronic myocarditis in humans (Savoia and Oxman, 1995). In order to study virus-specific protection and CVB3-caused pathogenesis in more detail, several murine models have been established (Chow et al., 1992 Huber and Pfaeffle, 1994 Henke et al., 1995 Mena et al., 1999). Despite the well characterized molecular structure of Coxsackie viruses (Natarajan and Johnson, 1998) and the successful use of common vaccinations in animal models (Fohlman et al., 1993 See and Tilles, 1997), no virus-specific preventive procedures against CVB3 are in clinical use today. A new method to prevent virus-caused disease is the administration of plasmid DNA encoding immunogenic viral epitops. The inoculation of DNA vaccines into muscle tissue or skin has been shown to induce humoral (Boyer et al., 1999 Konishi et al., 1999) as well as cellular (Schirmbeck et al., 1995; Qiu et al., 1999) immune responses and to confer protection against viral infections (Yokoyama et al., 1995 Operschall et al., 1999).

Using DNA plasmids encoding different sequences of CVB3 structural proteins we demonstrated that the expression of the capsid protein VP1 was the most effective vaccine to protect mice against a lethal CVB3 infection. In order to analyze the putative protective effect of the other

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single capsid proteins, VP2, VP3, and VP4 were cloned and several different DNA vaccines were established. We constructed the plasmids pCMV/VP2, pCMV/VP3, and pCMV/VP4, which encode the following sequences: VP2 (788 bp), VP3 (713 bp), and VP4 (206 bp), using the same method as described for pCMV/VP1 (Henke et al., 1998). Plasmid isolation from bacteria was performed using the endotoxin-free plasmid preparation kit (Qiagen, Hilden, Germany). Expression from these plasmids was determined in vitro by transient transfection of HeLa cells. After RNA isolation, DNase digestion, and reverse transcriptase reaction, the transcriptional activity of all plasmids was confirmed by PCR (data not shown).

After the expression from the DNA vaccines was analyzed in vitro, male BALB/c mice were inoculated intramuscularly (i.m.) twice in each quadriceps muscle separately with 100 μg of plasmid DNA at 4-week intervals. One group of mice remained untreated and one group received the parental vector pCMV as a negative control. Four weeks after the second immunization, mice were subjected to intraperitoneal (i.p.) challenge with a normally lethal dose of CVB3 (5 LD_{50}). After this challenge, the number of surviving animals was monitored up to 4 weeks post infection (p.i.). As it is shown in 1A, the most effective vaccine was pCMV/VP1, confirming the result we obtained before (72.2% protection) (Henke et al., 1998). In this study, the percentage of surviving animals using this vaccine was 71.4% (10 of 14). Intramuscular inoculations of all other plasmids were less effective, inducing incomplete protection of 21.4% (3 of 14 with pCMV/VP3) or 20% (2 of 10 with pCMV/VP2). Expression of the protein VP4 induced no protection (0 of 12 with pCMV/VP4). These results confirmed, that the major capsid protein VP1 — expressed as a single protein — is the best candidate among the capsid proteins to be used in plasmid DNA expression vectors reflecting the observation that several immunogenic epitopes are located within this protein (Haarmann et al., 1994). In other experimental animal models using different picornaviruses like the encephalomyocarditis virus (Jun et al., 1995) or the foot and mouth disease virus (Huang et al., 1999), the VP1 protein expressed as a single protein or partly expressed as peptides was found to be protective as well. In order to analyze the mechanism by which the expression of VP1 was able to induce a protective status in vaccinated mice, the following approach was used: male BALB/c mice were inoculated with pCMV as a control or vaccinated with pCMV/VP1 in the same way as described above. Three days after the lethal CVB3 challenge with 5 LD_{50} doses, the weight of the individual mice was determined. The

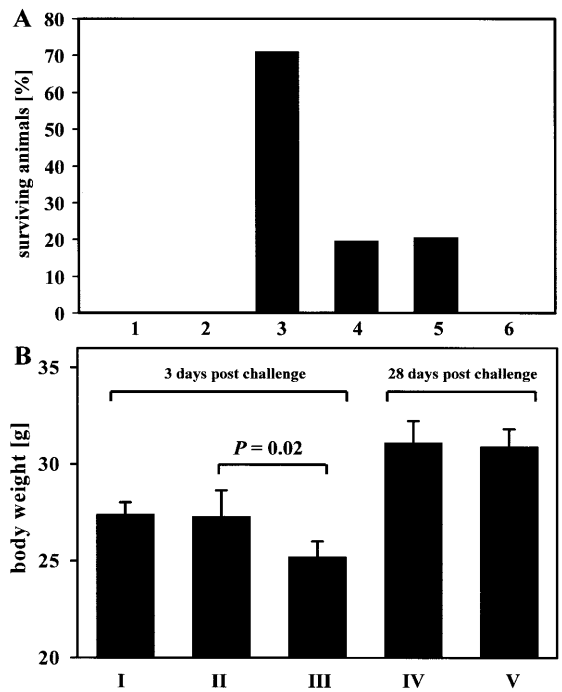

Fig. 1. Protection against a normally lethal CVB3 challenge induced by DNA vaccination. (A) BALB/c mice were immunized with plasmid DNA encoding either CVB3 VP1 (3), VP2 (4), VP3 (5), or VP4 (6) sequences, with the control plasmid pCMV (2); some remained untreated (1). Four weeks after the second inoculation, mice were challenged with 5 LD_{50} doses of CVB3 i.p. The percentage of surviving animals was monitored over a time period of 28 days. The results presented are summarized data of three independent experiments using at least three to five mice in each group. (B) Three days and 28 days after the lethal CVB3 challenge, the body weight of pCMV-treated control mice (III) was compared with the body weight of pCMV/VP1-vaccinated (II, V) and non-infected control mice (I, IV). The data represent the mean ± standard deviation using eight mice in each group. The statistical comparison was carried out with Microsoft Excel by using Students' *t*-test.

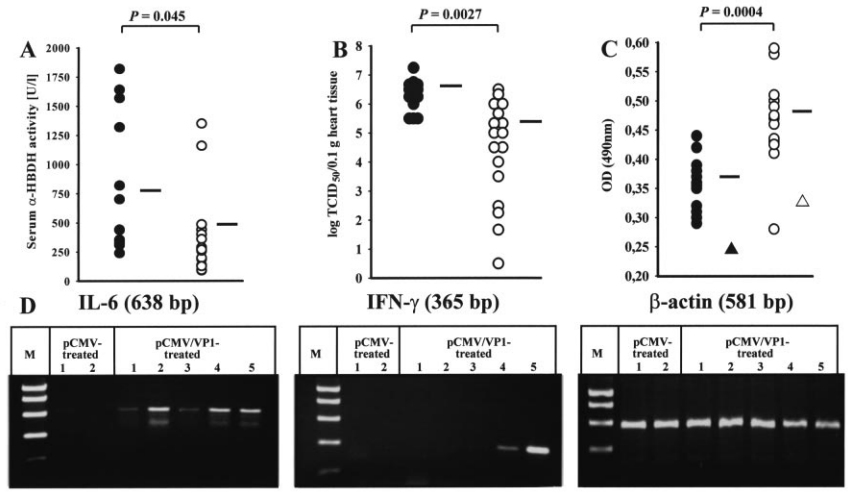

Fig. 2. Characterization of pCMV/VP1-mediated protection, 3 days after the lethal CVB3 challenge. The concentration of the cardiocyte-specific enzyme α-HBDH (A) and the amount of CVB3-specific antibodies (C) — detected by ELISA (serum dilution 1:25) and demonstrated as the optical density at 490 nm (OD_{490}) — were analyzed in sera of individual pCMV (●) or pCMV/VP1 (○) inoculated mice. The viral load in the heart tissue of individual mice was determined using $CCID_{50}$ assays (B). The black bar in each figure indicates the mean value. ▲ and △ indicate the mean value of CVB3-specific antibody titers before the lethal challenge. The transcriptional activity of IL-6 and IFN-γ was analyzed using individual heart tissue obtained from two pCMV- and five pCMV/VP1-inoculated mice by RT-PCR (D). The statistical comparison was carried out with Microsoft Excel by using Students' *t*-test.

significant loss of body weight of pCMV-treated mice indicated that these mice already had an ongoing infection, which is common in CVB3-infected mice at this time (1B, III). Mice which received the pCMV/VP1 vaccine remained in the same body weight range as normal non-infected control mice (1B, II and I). Four weeks after the CVB3 challenge, the body weight of surviving pCVM/VP1-immunized mice was identical to that of non-infected mice, indicating that no ongoing pathogenic process was present in these mice (1B, V and IV). At this time, no pCMV-inoculated mouse survived the lethal CVB3 challenge.

Three days after the CVB3 challenge, serum as well as heart tissue were obtained from plasmid DNA-inoculated mice. In sera, the concentration of the cardiocyte-specific enzyme α-hydroxybutyrate-dehydrogenase (α-HBDH) was analyzed using a commercially available kit (Sigma Diagnostics, St Louis, USA) according to the instructions of the manufacturer. Heart muscle cells contain high levels of α-HBDH and myocardial damage results in an increased release of this enzyme into the blood (Franken et al., 2000). α-HBDH activity was compared to the whole LDH (lactate dehydrogenase) activity (Sigma Diagnostics) demonstrating that the quotient LDH/α-HBDH was always below 1.3, which indicates heart tissue destruction (data not shown). In addition, no significant inflammation at the site of the DNA vaccine inoculation was observed at this time, indicating that only minor levels of α-HBDH might be contributed by skeletal myocyte damage. As is demonstrated in 2A, high levels of α-HBDH-activity were detectable in sera of pCMV-treated control mice 3 days after the challenge. In contrast, α-HBDH activity in the sera of pCVM/VP1-vaccinated mice was significantly decreased. Furthermore, the infectious virus was isolated from the heart tissue of the same mice as described previously (Henke et al., 1995) and the virus titer was determined by cell culture infectious dose 50% assays ($CCID_{50}$) on HeLa cell monolayers (2B). High levels of infectious virus were present in the pCMV-treated group and were accompanied by high concentrations of α-HBDH

activity in the sera. In contrast, decreased viral concentrations in the heart tissue caused significantly less damage in the myocardial cells of vaccinated mice. The heart tissue of surviving animals did not show histopathological disorders, indicating that CVB3 was unable to induce tissue destruction, massive inflammation, or fibrosis 45 days p.i., as has been demonstrated before (Henke et al., 1998). Furthermore, levels of immunoglobulin M (IgM)- and IgG-specific antibodies were assessed together by enzyme-linked immunosorbent assay (ELISA), using purified CVB3 as the target antigen. As is demonstrated in 2C, the vaccination procedure induced low levels of anti-CVB3 antibodies. However, 3 days after the challenge, a significant increased concentration of CVB3-specific antibodies was detectable in the sera of pCMV/VP1-vaccinated mice, indicating that the vaccination procedure induced an immunologic memory that was accompanied by a secondary antibody response. This may then lead to reduced viral replication in the heart tissue detected in the vaccinated mice. In addition, the activated immune response was also reflected by an early induction of the transcriptional activity of immunoregulatory cytokines in the myocardial tissue, indicating an early immune infiltration of the heart tissue. In general, cytokine transcription and translation were found to be highly increased in CVB3-caused myocarditis, as has been described by several different investigators (Huber et al., 1996 Gebhard et al., 1998 Opavsky et al., 1999). During our vaccination experiments we analyzed the activation of interleukin-6 (IL-6) and interferon-γ (IFN-γ) transcription in heart tissue 3 days after the lethal challenge. Briefly, cytokine expression was determined in individual tissue samples of pCMV- or pCMV/VP1-inoculated mice by RT-PCR. After RNA isolation, DNase digestion, and reverse transcriptase reaction, the transcriptional activity of IL-6, as well as that of IFN-γ, was confirmed by PCR and compared to β-actin expression (2D). At this time point, only in pCMV/VP1-vaccinated mice was cytokine expression detectable, indicating an early activation of immunoreactivity against the viral infection.

Furthermore, the activation of the cellular immune response in pCMV/VP1-vaccinated mice was characterized by using a VP1-expressing recombinant vaccinia virus (VV/VP1) to infect target cells. The reason for using this approach is that genetically well-defined murine target cells are normally not susceptible to infections with CVB3. Therefore, the recombinant VV/VP1 was established as described before (Henke et al., 1998 Wessely et al., 1998). BALB/c mice were plasmid-inoculated using the same schedule as described above. Four weeks after the second immunization, spleen cells were obtained from pCMV/VP1- and pCMV-treated mice and a non-radioactive cytotoxic T lymphocyte (CTL) assay was performed detecting the release of LDH in cell culture supernatants according to the instructions of the manufacturer (Boehringer, Mannheim, Germany). For this assay, murine Balb Cl 7 (H-2^d) target cells as well as MC57 (H-2^b) target cells (Klavinskis et al., 1990) were infected with VV/VP1, but no CVB3-specific CTL activity was observed (data not shown). Therefore, pCMV/VP1-vaccinated as well as pCMV-treated control BALB/c mice were challenged with 5 LD_{50} doses of CVB3 and 3 days later, spleen cells were obtained and used as effector cells for CTL assays. Under these test conditions, only Balb Cl 7 target cells were lysed, indicating MHC-class I-restricted CTL activity of spleen cells obtained from pCMV/VP1-vaccinated BALB/c mice, whereas no lysis was detectable using MC57 target cells. The specific lysis observed was almost in the same range as the CTL activity detectable from spleen cells obtained from BALB/c mice infected twice with a 0.1 LD_{50} dose of CVB3 (Fig. 3). The CTL activity of these spleen cells was analyzed 3 days after the second infection, whereas no CTL activity was observed using spleen cells from mice after a single infection (data not shown).

In conclusion, we have demonstrated here, that the CVB3 capsid protein VP1 conferred protection of 71.4%, whereas VP2 and VP3 were less effective and VP4 failed to prevent CVB3-caused disease. Therefore, the following experiments were focused on the characterization of the vaccine-mediated protection induced by the plasmid pCMV/VP1. Three days after the lethal CVB3 challenge in the heart tissue of vaccinated mice: (i) CVB3-

caused myocyte destruction was less severe, (ii) the number of infectious virus particles was reduced, and (iii) increased levels of CVB3-specific antibodies were detectable by ELISA, indicating induced immunologic memory in vaccinated mice in comparison to control mice. These vaccine-mediated changes were accompanied by an early expression of immunoregulatory cytokines in the heart tissue of vaccinated mice and increased virus-specific cytotoxic activity of spleen cells. Further experiments will be focused on suitable methods to increase the efficiency of the established DNA vaccine pCMV/VP1.

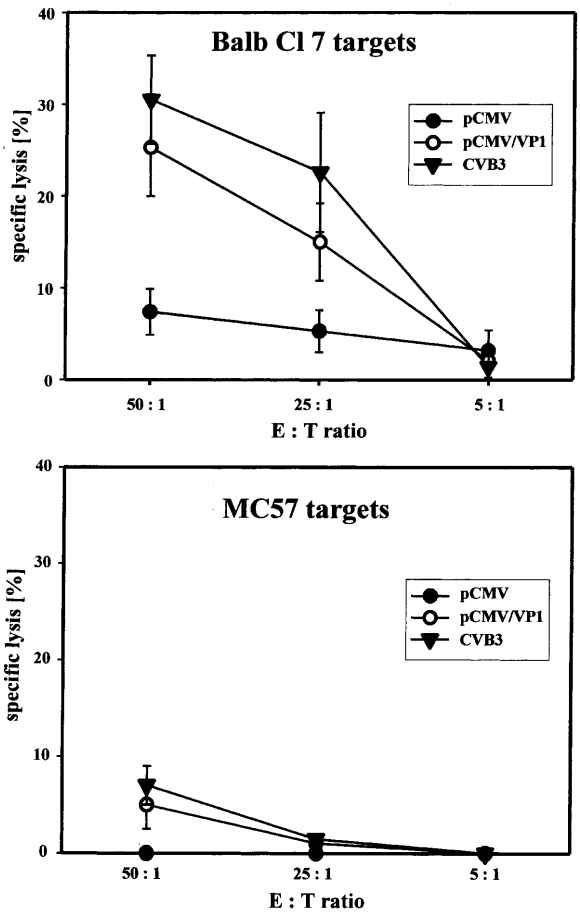

Fig. 3. Characterization of the cellular immune response in pCMV/VP1-vaccinated mice. Three days after the lethal CVB3 challenge, the CTL activity of spleen cells obtained from pCMV (●) or pCMV/VP1 (○) inoculated mice was characterized. Murine Balb Cl 7 as well as MC57 target cells were infected with a CVB3 VP1-expressing recombinant vaccinia virus (VV/VP1) and MHC class I-restricted CTL activity was analyzed using a non-radioactive CTL assay. The effector target cell ratio was between 50:1 and 5:1. Spleen cells — obtained from CVB3-infected BALB/c mice, 3 days after the second infection — were used as a positive control (▼). The data represent the mean ± standard deviation using five mice in each group.

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