Anti-coxsackievirus B4 (CV-B4) enhancing activity of serum associated with increased viral load and pathology in mice reinfected with CV-B4

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ABSTRACT

In previous studies it was shown that inoculation of Swiss albino mice with CV-B4 E2 resulted in the production of serum IgG capable of enhancing the CV-B4 E2 infection of murine spleen cells cultures. To investigate whether such an enhancing activity of serum can play a role in vivo, we decided to study the CV-B4 E2 infection in mice exposed to successive inoculations of virus. In Swiss albino mice infected with CV-B4 E2 at the age of 21 days, anti-CV-B4 E2 neutralizing and enhancing activities of their serum peaked after 55 d. In contrast, mice inoculated at the age of 55 d expressed much lower activities. Despite the neutralizing activity of serum, CV-B4 E2 inoculated a second time to 55 day-old animals spread into the host. At the age of 72 and 89 d the levels of viral RNA and infectious particles were higher in organs of animals exposed to 2 successive infections compared with animals infected once at the age of 21 d or 55 d. In animals with 2 successive inoculations of CV-B4 E2 there was a relationship between the anti-CV-B4 E2 enhancing activity of serum and the level of viral RNA in organs and an enhancement of pathology was observed as displayed by histological analysis of pancreas and hyperglycaemia. Altogether our data strongly suggest that an anti-CV-B4 E2 enhancing activity in the host can play a role in the outcome of a secondary infection with this virus.

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Introduction

The Enterovirus genus encompasses many human serotypes distributed in 7 species Enterovirus A to D and Rhinovirus A to C. They are small, non-enveloped, positive single-stranded RNA viruses belonging to the Picornaviridae family. Type B Coxsackievirus (CV-B) encompassing CV-B 1–6, may be responsible for a large pathological specter varying from benign acute to severe chronic diseases such as chronic myocarditis, dilated cardiomyopathy, and type 1 diabetes. An association between enteroviruses, especially CV-B and type 1 diabetes (T1D) was reported. Enteroviral components have been more frequently detected in blood of T1D patients, and a meta-analysis has reported a strong association between EV detection, especially CV-B, and T1D. The presence of EV protein in the pancreas of T1D patients has been observed. Prospective studies confirmed the link between EV and the development of pancreas islet autoimmunity. Moreover, experimental studies, in vitro and in vivo animal models, revealed that CV-B4 may be involved in the pathogenesis of T1D through several mechanisms.

It has been shown that non-neutralizing anti-CV-B4 IgG obtained from serum of patients can increase the infection of peripheral blood mononuclear cells (PBMC) with CV-B4, which results in production of IFNα and other inflammatory cytokines. The role of enhancing IgG levels in the outcome of CVB4 infections and other conditions such type 1 diabetes, cannot be ignored. However the impact of anti-CV-B4 enhancing activity of serum/IgG in the infection with this virus, in vivo, remains to be investigated.

A variant of CV-B4, called CV-B4 E2, isolated from a patient who died after onset of type 1 diabetes, was able to induce diabetes in mice. It was reported that this diabetogenic strain CV-B4 E2 could infect outbred Swiss albino mice and induce a diabetes-like clinical phenotype. The aim of the present study was to determine if an enhancing activity of serum/IgG in the infection with this virus, in vivo, remains to be investigated.

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Samples of the 7 organs were treated to extract ARN and to evaluate the level of enteroviral RNA by quantitative real-time RT-PCR and to isolate infectious particles. The detection of infectious particles and of viral RNA in serum at the age of 38, 55, 72 and 89 d was negative in animals inoculated with the virus at the age of 21 d or 55 d or both (data not shown).

In mice inoculated at the age of 21 day-old (group 1), the anti-CVB4 E2 neutralizing and enhancing activities in serum peaked when they were 55 day-old as expected following the results obtained in the first part of this study (Fig. 2a and 2b). Viral RNA was detected in the first sample (collected at the age of 38 days) and in the next one (at the age of 55 days) in each organ afterwards the levels

![Figure 1. Continued](image-url)
Figure 1. Individual follow-up representation of anti-CV-B4 E2 neutralizing and enhancing activity of serum from CV-B4 E2-inoculated mice. Blood samples were successively collected from 10 mice inoculated with $2 \times 10^4$ TCID50 CV-B4 E2 at the age of 21 d (cross), and 10 mice inoculated at the age of 55 d (square). Serum was recovered and each serum sample was tested for neutralizing activity (a) and enhancing activity (b and c). 

a) Neutralizing activity. Neutralizing titers of serum samples were defined as the reciprocal of the last dilution that totally inhibited the CVB4 E2-induced CPE in HEp-2 cell cultures.

b) Enhancing activity tested through the antiviral activity in supernatants of spleen cells cultures inoculated with CV-B4 E2 mixed with serum from CV-B4 E2-infected mice. Serum samples were diluted at a ratio of 1:500 and mixed with CV-B4 E2. The mixture was then inoculated to murine spleen cells cultures (MOI = 0.02) and supernatants were harvested after 48 hours of incubation. The antiviral activity of supernatants was tested using a bioassay. The antiviral titers of supernatant samples were defined as the reciprocal of the last dilution that totally inhibited the EMCV-induced cytopathic effect in L-929 cell cultures.

c) Enhancing activity tested through the quantification of intracellular viral RNA in spleen cells cultures inoculated with CV-B4 E2 mixed with serum (diluted 1:1,000) from CV-B4 E2-infected mice. Total RNA was extracted and the level of viral RNA was determined by real time QPCR. The results were expressed as viral RNA copy number per ng of total RNA.
decreased dramatically or were null. The maximum values were lower than $10^2$ in most organs and lower than $10^3$ in the heart. Infectious particles were isolated from every organ in agreement with the detection of viral RNA. The levels of infectious particles expressed as PFU per mg peaked in organ samples collected when mice were 55 day-old (or 72 day-old in the case of pancreas), the values ranged from $10^2$ to $10^4$, which decreased to less than $10^1$ in brain, spinal cord and thymus samples obtained from mice at the age of 89 day-old whereas in other organs (pancreas, intestine, heart and spleen) at this age the values were around $10^3$ (Fig. 2c).

Figure 2. Continued
In mice inoculated with CV-B4 E2 at the age of 55 day-old (group 2), the anti-CVB4 neutralizing and enhancing activities in serum were very low (<32 and <2 respectively) which is in agreement with the results obtained in the first part of this study (Fig. 2a and b). Viral RNA was detected in the first sample (collected at the age of 72 days) and in the next one (at the age of 89 days) in pancreas and spleen, the values were < 10^2. Viral RNA was also detected in heart and thymus, the values were < 10^3, but was not detected in brain, spinal cord and intestine. The levels of infectious particles were low < 10^2 in spleen, pancreas and heart obtained from those animals at the age of 72 and 89 day-old, and were lower in other organs in agreement with the viral RNA values (Fig. 2c).

In mice inoculated at the age of 21 d with CV-B4 E2 and inoculated a second time at the age of 55 d (group 3), the anti-CV-B4 E2 neutralizing activity peaked at the age of 72 d (1638 ± 560 and then decreased at the age of 89 d (921 ± 163) (Fig. 2a). At these ages the enhancing activity of serum was high as shown by the level of viral RNA in spleen cells cultures inoculated with mixtures of CV-B4 E2 and serum samples. The values were much higher than those obtained with serum from mice at the same ages that were infected once at 21-day old (2.5 10^7 ± 900 vs 6.10^3 ± 1000 at the age of 72 d p < 0.05; 1.4 10^7 ± 800 vs 6.10^2 ± 160 at the age of 89 d p < 0.05). Viral RNA was detected in each organ, and the levels at the age of 72 d and 89 d in mice inoculated twice with CV-B4 E2 (group 3) were higher.

Figure 2. Biological activity of serum from mice inoculated once or twice with CV-B4 E2 and viral parameters in their organs. Female Swiss Albino mice were inoculated with CV-B4 E2 at the age of 21 d (group 1), 55 d (group 2), or inoculated successively at the age of 21 d and 55 d (group 3). Each inoculum was 2 10^4 TCID50. Group 1 and group 2 encompassed 25 animals each, while group 3 encompassed 15 animals. Five animals were sacrificed at the ages of 21, 38, 55, 72 and 89 d (groups 1 and 3) and at the ages of 55, 72 and 89 d (groups 2). a – a”) Neutralizing activity of serum samples (see legend of figure 1). The results are mean ± SD, n = 5. b – b”) Enhancing activity tested through the quantification of intracellular viral RNA in spleen cells cultures inoculated with CV-B4 E2 mixed with serum (see legend of figure 1). The results are mean ± SD, n = 5. c – c”) Viral parameters in organs. A sample of each organ collected in mice inoculated with CV-B4 E2 was processed as described in the materials and methods section to determine the level of viral RNA expressed as copy number per ng of total RNA (black bars) and the level of infectious particles expressed as pfu/mg (white bars). The results are mean ± SD, n = 5. Arrow: CV-B4 E2 inoculation. NS: not significant (p value type = “Other” > 0.05). The data obtained at the age of 72 and 89 d in mice inoculated once with the virus at the age of 21 d or inoculated twice with the virus at the age of 21 d and 55 d were analyzed. In most cases values obtained in mice inoculated twice were significantly higher than those obtained in mice inoculated once. Only the not significant (NS) differences are indicated.
Figure 3. (For figure legend, see page 915.)
than those obtained at the same age in heart, pancreas, spleen and intestine of mice inoculated once at the age of 21 d (group 1) p values ranging from 0.022 to < 0.001. As far as the other organs the pattern of results at the ages of 72 d and 89 d were not so clear; p values were 0.057 and 0.001 respectively in the case of thymus, < 0.01 and 0.06 in the case of brain, and 0.4 and 0.045 in the case of spinal cord. Nevertheless, the viral RNA levels in all organs were much higher than those obtained at the same age in organs of mice inoculated once at the age of 55 d (group 2) (p values < 0.05 (Fig. 2c).

The levels of infectious particles in heart, pancreas, intestine, spleen and thymus obtained at the ages of 72 and 89 d from mice inoculated twice were higher than in those organs obtained at the same ages from mice inoculated once (p value < 0.01). In contrast there was no significant difference in the levels of infectious particles in the brain and spinal cord in animals which were inoculated once or twice with the virus (see Fig. 2).

**Relationship between the anti-CV-B4 E2 enhancing activity of serum and the level of viral RNA in organs**

Viral RNA was detected in organs of mice inoculated at the age of 21 d with lower levels than mice inoculated successively at the age of 21 and 55 days, whereas they were very low or null in organs of mice inoculated at the age of 55 d. In mice inoculated later in life the enhancing activity of their serum was very low or null compared with animals inoculated at the age of 21 d or inoculated twice. These observations prompted us to study the relationship between the serum enhancing activity and the level of viral RNA in organs.

The enhancing activity of serum measured through the viral RNA copy number in spleen cell cultures, inoculated with CV-B4 E2 mixed with serum (diluted 1 /1000), was compared with the level of viral RNA in organ samples in animals infected at the age of 21 d. A significant correlation, (p value < 0.0001), between serum activity and intestinal viral load was only obtained when the animals were 72 day-old (Fig. 3a). However there was no correlation between serum activity and intestinal viral load or other organs (brain, spinal cord, spleen and thymus, heart and pancreas) when the animals were 38, 55, 72 or 89 day-old (data not shown).

When the parameters were compared in animals infected successively at the age of 21 and 55 days, a significant correlation was obtained between serum activity and viral load in spleen (p value < 0.0001) and intestine (p value < 0.0001), collected when the animals were 72 day-old, and viral load in heart and pancreas collected when the animals were 72 day-old and 89 day-old as well (p value < 0.003) (Fig. 3b). However no correlation was observed between serum activity and viral load in brain, spinal cord and thymus when collected at the same age (data not shown).

A positive correlation was obtained between the anti-CV-B4 E2 enhancing activity of serum and the viral load in organs at the age of 72 d and/or 89 d, whereas there was no relationship between the anti-CV-B4 E2 neutralizing activity of serum and the level of viral RNA in organs (data not shown).

The relationship between the anti-CV-B4 E2 serum activity, enhancing and neutralizing, and the level of infectious particles isolated from organ samples was studied. There was no significant positive correlation between these parameters in animals inoculated once at age of 21 d or inoculated successively at the age of 21 and 55 d (data not shown).

**Enhanced pathology in mice inoculated with CV-B4 E2 at 21 d and 55 d of age successively**

There was no evidence of heart, pancreas (Fig. 4a) and brain damage in control mice (n = 5) and in mice inoculated once with CV-B4 E2 at the age of 55 d (n = 5). There was no lesion of organs in mice inoculated at the age of 21 d when they were 38 (n = 5) and 55 day-old (n = 5) (data not shown). Little or no inflammation was observed in heart from one out of 5 mice at the age of 72 d and in pancreas from 2 out of 5 animals at this age (Fig. 4b) and from one out of 5 at the age of 89 d (data not shown).

In marked contrast loss of exocrine tissue replacement with fat tissue and infiltration of mononuclear cells were observed in pancreas from 5 out of 5 mice at the age of 72 d (Fig. 4c) and from 3 out of 5 mice at the age of 89 d (data not shown) when the animals were inoculated successively at the age of 21 and 55 d. An extensive
infiltration of mononuclear cells was seen in the heart collected at the age of 72 d from one out of 5 mice inoculated twice with CV-B4 E2 (Fig. 4c).

In contrast, there were no apparent lesions in the brain at the age of 72 or 89 d in any animal inoculated once or twice with CV-B4 E2 (data not shown).

To investigate further the impact of CV-B4 E2 onto the host, we measured the serum levels of glucose in animals as described in the materials and methods section. In animals infected twice, the serum levels of glucose at the age of 72 and 89 d were 439 +/- 80 mg/dl (n = 5) and 393 +/- 56.72 mg/dl (n = 5) respectively and were

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**Figure 4.** Histologic examination of organs from mice. Female Swiss Albino mice were inoculated with culture medium (a), or CV-B4 E2 when they were 21 d old (b), or inoculated successively when they were 21 and 55 d old (c). Each inoculum was 2 10^4 TCID50. Animals were sacrificed and organs were collected and processed as described in the material and methods section. The slices were mounted and observed under Zeiss Axioscan(Z1) Scanner. The pictures represent pancreas and heart obtained from animals at the age of 72 d. The arrows show inflammatory foci.
significantly higher than the values measured at the same age in serum of animals infected only once at the age of 21 d (see Fig. 5). Indeed the mean levels of glucose in serum from 21 through 89 d of age in these animals were lower than 200 +/- SD mg/dl. The pattern of results was similar in serum from mice inoculated when they were 55 day-old and in control animals from 21 through 89 d of age with mean values ranging from 162 +/- 54 to 221 +/- 39 mg/dl (see Fig. 5).

Discussion

The current study addressed the issue of successive infections with CV-B4 E2 in mice.

Several considerations are noteworthy. Recently our team reported that serum samples from mice inoculated with CV-B4 E2 enhanced the infection of spleen cells with this virus. In our previous report the infection of spleen cells inoculated with CVB4 mixed with diluted immune serum was assessed by the detection of viral RNA by RT-PCR, whereas viral RNA was only detected by sn-RT-PCR, a more sensitive technique, when cells where inoculated with CV-B4 E2. In the present study, the levels of intracellular viral RNA in spleen cells were measured by quantitative real-time RT-PCR. The quantification of viral RNA in this bioassay displayed the enhancing activity of diluted serum and enabled evaluation of the extent of this property in each serum sample obtained successively up to the age of 89 d. Moreover the quantitative evaluation of the enhancing activity of serum samples allowed inter-individual comparisons and allowed the investigation of the relationship between serum enhancing activity and the level of viral RNA in organ samples.

This study shows that mice infected at the age of 21 and 55 d have higher levels of viral RNA and infectious particles at the age of 72 and 89 days, as proved by the higher values of viral parameters in these mice when compared with mice infected only once at the age of 21 d. The level of viral markers in mice infected twice is not due to the sum of the parameters observed at the age of 21 and 55 days, the latter case exhibiting very low values.

The level of viral RNA in our experiments was expressed as copy of viral RNA / ng of total RNA in tissue. Considering the amount of RNA in 1 mg of tissue the level of viral RNA per mg of tissue was highly

Figure 5. Glucose levels in serum. Female Swiss Albino mice were inoculated with CV-B4 E2 when they were at the age of 21 d (a), 55 d (b), or inoculated successively when they were 21 and 56 d (c). Each inoculum was 2 x 10^5 TCID50. Uninfected animals served as controls (d). In each case 5 animals were sacrificed at the age indicated in the x axis. Blood was collected; serum was obtained and stored at -80°C. Then the serum levels of glucose were measured on a glucometer as described in the materials and methods section. The results are means +/- SD (n = 5). *p value 0.001 **p value 0.02 vs serum level of glucose in mice at the age of 72 and 89 d infected once at the age of 21 d (a).
multiplied (> 100). Thus the amount of viral RNA compared with the level of PFU per mg of tissue is in agreement with the fact that picornaviruses produce an excess of RNA compared with infectious particles. There was no correlation between the levels of viral RNA and infectious particles in organ samples in our experiment. We have no clear explanation; a discrepancy between these parameters can be due to a poor recovery rate of infectious particles and/or viral RNA. Moreover, it cannot be ruled out that neutralizing specific antibodies in the samples, at high levels in certain individuals, as reflected by the results of sero-neutralization assays, impaired the isolation of infectious particles, even though there was no inverse correlation between the anti-CV-B4 E2 neutralizing titres and the amounts of isolated infectious particles (data not shown).

In mice infected at a late stage, at the age of 55 days, the levels of viral RNA and infectious particles in organs were very low compared with mice infected at an earlier stage at the age of 21 d. The moderate infection in older mice was not due to a stronger immune response resulting in a more efficient viral clearance since the level of anti-CV-B4 E2 neutralizing activity in their serum was lower than in other mice infected at the age of 21 d. This pattern of CV-B4 E2 infection in older mice can be due to a natural resistance to the virus at late stages of life possibly because the levels of the virus receptor, the coxsackievirus and adenovirus receptor (CAR) falls significantly with age. Interestingly, however, in older mice infected with CV-B4 E2, it has been observed that the anti-CV-B4 enhancing activity of their serum was very low. Whether this pattern of enhancing activity plays a role in the low viral load in older animals remains an open question, but this observation suggests that most probably successive viral infections in older mice would not result in high viral loads in organs and consequently young individuals, with a higher enhancing activity of their serum, are more prone to an increased viral load following repeated infections. Furthermore as CAR is known to be upregulated in stressed tissues, an increased expression of CAR following the first infection may to some extent participate in the enhanced viral titers in these tissues observed in reinfected mice.

It is intriguing that 21 day-old mice inoculated with CV-B4 E2 were successfully infected a second time at the age of 55 days, as proved by the high viral load in organs in the following days, even though the anti-CV-B4 E2 neutralizing activity of serum was strong when the virus was administered. This pre-existing neutralizing activity boosted by the second virus inoculation did not prevent the production of the virus as proved by the isolation of viral particles from organs in our experiments. Altogether, our data suggest that the anti-CV-B4 E2 enhancing activity in an individual can play a role in the outcome of a secondary infection with CV-B4 E2 in the host. Indeed, as demonstrated in this study, serum samples from 55 day-old mice that were infected at age of 21 d were able to enhance dramatically the infection of cells with CVB4 in vitro. Moreover, a relationship between the anti-CV-B4 E2 enhancing activity of serum, and the levels of viral RNA in organs collected at the age of 72 and 89 d from mice infected twice was observed. Thus it cannot be excluded that under favorable condition, reflected by the anti-CV-B4 E2 enhancing activity of serum, a secondary exposure to CV B4 E2 result in a successful infection with a high viral load in organs despite a strong neutralizing activity of serum.

It can be assumed that the anti-CV-B4 E2 enhancing activity of serum displayed in vitro was due to anti-CV-B4 E2 IgG as reported in our previous studies. Thus, such IgG were present in serum samples from mice inoculated with CV-B4 E2 but was not detected in their serum before exposure to the virus as shown in the present study when the individual anti-CV-B4 E2 enhancing activity was tested in consecutive serum samples from 10 animals. These observations strongly suggest that the anti-CV-B4 E2 enhancing activity of serum was due to anti-CV-B4 E2 antibodies, and that these antibodies played a role in the pattern of infection of animals in vivo. Further studies are needed to investigate whether anti-CV-B4 E2 antibodies are involved in the increased level of viral markers in animals inoculated with the virus, and particularly in mice infected with CV-B4 E2 successively at the age of 21 d and 55 d in our experiments.

Histological examination of tissue from mice in our experiments showed lesions of pancreas, and of heart but at a lower extent, that were more prevalent and/or pronounced in samples obtained from 72-day-old mice infected successively at the age of 21 and 55 d compared with samples obtained from mice at the same age, or at the age of 38 and 55 days, infected only once at the age of 21 d.

This impact of CV-B4 E2 onto the tissues in mice inoculated twice was surprising. Indeed, it was expected that mice with high anti-CV-B4 E2 neutralizing activity in serum were protected against lesions of organs when the virus was inoculated a second time. It cannot be excluded that the neutralizing activity in this case limited the spreading and/or the impact of the virus. Nevertheless the animals were not protected against lesions of organs in these conditions. The extent of the lesion was high in pancreas of mice inoculated twice with CV-B4 E2; whereas in brain there were no apparent injuries. In the pancreas, but not in the brain of mice inoculated
twice, the viral load was high and related to the enhancing activity of serum. Altogether this data suggests that lesions of the pancreas were related to the viral load in tissues, the extent of which depended on enhancing antibodies.

The serum level of glucose measured on a glucometer was high in mice at 72 and 89 d of age, infected twice with CV-B4 E2 (> 300 mg/dl) whereas there was no difference between serum levels of glucose in mice infected only once and controls groups (around 200 mg/dl). The glycaemia in mice was already measured on a standard glucometer, and animals were considered hyperglycemic when readings were greater than 200 mg/dl in previous studies. In these studies the glucose levels were measured using blood obtained from the tail vein or eye bleeds, in contrast our study used serum samples obtained from blood of sacrificed animals. There is a discrepancy between our results and those of other authors regarding the impact of CV-B4 E2 onto the glucose homeostasis.

In our experiments, hyperglycemia was expressed in female Swiss albino mice exposed to CV-B4 E2 twice, but there was no disturbance from 17 d through up to 70 d post-inoculation in these mice challenged once intraperitoneally with $2 \times 10^4 \text{TCID}_{50}$ at the age of 21-day-old. In contrast, in the previous study hyperglycemia was observed starting from 49 d after infection of male Swiss-derived CD1 mice with $10^4$ pfu CV-B4 E2 when they were 28–35-day-old. In the previous study CV-B4 E2 stocks were prepared in monolayers of monkey kidney cells whereas in our experiments virus stocks were prepared in human HEp-2 cell cultures. Whether such differences, in conjunction with the type and gender of mice, can explain the discrepancy remains to be determined.

It was reported that in 6-week-old SJL mice, a homologous challenge intraperitoneally with $10 \times 4\text{ pfu CV-B4 E2}$ 6 weeks after the first inoculation resulted in an enhancement of hyperglycaemia independently of the T cell response. This could be due to an antibody-mediated enhancement of infectivity though antibodies able to increase the virus spreading and replication resulting in hyperglycaemia as suggested by the relationship between the anti-CV-B4 E2 enhancing activity of serum, the levels of viral RNA in organs, especially the pancreas, and the disturbance of glycaemia homeostasis observed in mice inoculated twice in the current study.

Based on the results of our study, it cannot be ruled out that an anti-CV-B enhancing activity in the host played a role in enhanced pathology observed in heterologous CV-B challenge of mice as previously reported.

The relationship between the anti-CV-B4 E2 enhancing activity of serum and the levels of viral RNA in organs, combined with the observation of enhanced pathology as displayed by tissue lesions and hyperglycaemia in mice challenged with CV-B4 E2 in the current study, suggest that an antibody-mediated enhancement of CV-B4 E2 infectivity could increase the virus spreading and replication resulting in a more severe outcome of the infection. Based on this hypothesis, the anti-CV-B4 enhancing activity in serum of human beings previously described by our team could be considered as a risk factor for enhanced pathology in case of exposure to CV-B4. Similarly an enhancing activity toward any CV-B would be a risk factor for enhanced pathology in case of homologous, and possibly, heterologous challenge with CV-B. Interestingly, in patients with type 1 diabetes, an anti-CV-B4 enhancing activity of serum was observed. Whether enhancing antibodies play a role in the pathogenesis of CV-B-induced diseases in vivo, especially type 1 diabetes deserves further studies. Future studies will be directed along this line in our laboratory.

**Material & methods**

**Virus and cells**

The diabetogenic strain CV-B4 E2 (kindly provided by J. W. Yoon, Julia McFarlane Diabetes Research Center, Calgary, Alberta, Canada), was propagated in HEp-2 cells (BioWhittaker, Vervier, Belgium) in Eagle’s minimal essential medium (MEM) supplemented with 10% fetal calf serum, 1% L-glutamine (Gibco BRL), 50 μg/mL streptomycin, and 50 IU/mL penicillin (Invitrogen, Saint Aubin, France). The *encephalomyocarditis virus* (EMCV) strain (ATCC) was propagated in L-929 cells (kindly provided by T Jouault, Lille, France) in Dulbecco’s modified eagle’s medium (DMEM; Gibco BRL, Invitrogen) supplemented with 10% fetal calf serum, 1% L-glutamine, 50 μg/mL streptomycin, and 50 IU/mL penicillin. Supernatants were collected 3 d after inoculation, clarified by centrifugation at 2,000 × g for 10 min divided into aliquots, and stored at −80°C.

Virus titers in stocks were determined on HEp-2 cells (CV-B4 E2) or L-929 cells (EMCV) by limiting dilution assay for 50% tissue culture infectious doses (TCID$_{50}$) and expressed as log$_{10}$ TCID$_{50}$/mL as described elsewhere.

**Animals**

All animals were handled by respecting the standards of general ethics guidelines, and maintained in specific
“pathogen-free” conditions with unlimited access to sterile food and water. All experiments were conducted following the guidelines of 2010 EU directory and were approved by Faculty of Pharmacy University of Monastir (Tunisia) and by the Ethical Committee for Animal Experimentation of Nord-Pas-de-Calais (France).

Three -week-old Female Swiss albino mice (Institut Pasteur, Tunis) were intraperitoneally inoculated with 200 μL culture medium or with CV-B4 E2 at 2 × 10⁴ TCID₅₀ units contained in 200 μL culture medium. Mice were followed up to the age of 89 d. When appropriate, blood samples were obtained from the tail vein of animals, using a needle. Serum samples obtained following blood samples were stored at -80°C and centrifugation of blood were stored at -80°C.

Other mice followed up to the age of 89 d were sacrificed using isoflurane at the ages of 21, 55, 72 and 89 d to collect blood and organs (heart, pancreas, intestine, spleen, thymus, brain and spinal cord). Serum samples obtained from blood were stored at -80°C. A part of the organ sample was stored at -80°C for investigating the level of viral RNA and infectious particles, and another part was fixed in formalin for histological study.

Seroneutralization assay

A modified Seroneutralization assay was used to assess the anti-CV-B4 E2 activity of mice sera. HEp-2 cells were first seeded at 10⁵ cells per well, in 96-well microtiter plates (Falcon, Oxnard, CA, USA) which were placed at 37°C in a humidified incubator with 5% CO₂. After 16 h of incubation, the medium was discarded and 50 μL of culture medium was added in each well. Then 5 μL of MEM or mouse serum samples, mixed with 45 μL of MEM (1:10 dilution to prevent cytotoxicity), was added to the first wells, and 2-fold serial dilutions were performed. Each sample was tested in triplicate. Afterwards, 25 μL of culture medium containing 25 TCID₅₀ CV-B4 and 25 μL of medium culture were added to each well and the plates returned to the incubator. The cell layers were examined 48 hr later under an inverted microscope (Olympus CKX41) at the objective lens 10x. The results were expressed as the inverse of the final dilution that inhibited the viral cytopathic effect.

Spleen cells cultures

The CV-B4 E2 enhancing activity of serum from mice was tested in murine spleen cells cultures as previously described.¹⁶ Spleens were aseptically removed from 5-week-old male Swiss albino mice (Janvier Laboratories, France) sacrificed by cervical dislocation. Spleen cells were prepared on ice, depleted of erythrocytes by hypotonic shock, then suspended in RPMI-1640 (Eurobio, Paris, France) supplemented with 10% FCS, 1% L-glutamine, 50 μg/mL streptomycin, 50 IU/mL penicillin and 10⁻⁵ M β-mercaptoethanol (Sigma). Cells were then seeded into 96-well culture plates at 5 × 10⁵ cells per well, and incubated at 37°C in a humidified atmosphere with 5% CO₂.

Mouse serum samples (5 μL) were diluted at 1:1000 in duplicate in supplemented RPMI and mixed (2/1; vol/vol) with CV-B4 E2 suspension (10⁵ TCID₅₀/mL). The mixtures were incubated for 2h at 37°C, then 85 μL of these mixtures were inoculated in duplicate to spleen cells cultures (5 × 10⁵ in 100 μL/well) and 100 μL of supplemented medium was added before incubating the microplates for 48 h. Afterwards, culture supernatants were collected, clarified and stored at -80°C for antiviral activity titration. Furthermore, spleen cells cultures were washed 5 times with cold phosphate buffered saline (PBS) and then prepared for RNA extraction followed by real-time QPCR to quantify the level of intracellular viral RNA.

Bioassay for evaluating the antiviral activity of supernatants of spleen cells cultures

The antiviral activity of supernatants of spleen cells cultures was evaluated using a bioassay based on the protection of L929 cells against the cytopathic effect (CPE) induced by EMCV as previously described.¹⁴ The inverse of the highest dilution providing 100% protection of the cells from EMCV-induced CPE was considered as the endpoint for antiviral activity.

Detection of infectious particles

Infectious particles were isolated from organ samples and the amount was evaluated as previously described.¹⁹ Briefly, snap frozen tissues were weighed and crushed using a tissue ruptor (Qiagen) in 1 ml PBS (1% penicillin/streptomycin), and then centrifuged at 2000 g for 10 min at 4°C. Supernatants were 10-fold diluted in MEM with 2% FCS and 500 μL and were inoculated to confluent HEp-2 cell layers in 6-well plates (Falcon, Oxnard, CA, USA) and 500 μL was used for RNA extraction. After 24 h of incubation at 37°C in a humidified incubator with 5% CO₂, the medium was discarded and 200 μL of MEM containing crushed organs samples (dilution 10⁻¹ to 10⁻⁹) were added to each well, and then the plates were incubated for 3 hours. Afterwards the supernatants were discarded and the cell layers were washed 5 times with culture medium, and then overlaid with culture medium containing agarose (1:10 dilution). The plates were kept at room temperature until the agar overlay turned solid. The plates were incubated, after 3–5 days, 1 mL of formaldehyde was added and 2h latter at
room temperature, the agarose overlay was discarded before staining the cell layers with crystal violet. The plaques were counted and the results were expressed as means of plaque forming units (PFU)/mg of tissue.

Quantification of viral RNA

RNA extraction

RNA was extracted from mice sampled tissues as described above and from 3 × 10⁶ spleen cells by the acid guanidium thiocyanate-phenol-chloroform extraction procedure by using Tri-Reagent (Sigma), as described elsewhere. Purified water for injection (C.O. M Laviosier) was submitted to the same extraction procedure and served as negative control. Supernatant of CV-B4 E2-infected HEp-2 cells served as positive control. Extracted RNA was dissolved in 50 µl of nuclease-free water (Promega), quantified using the Quant-iT RiboGreen RNA assay kit (Molecular Probes, Invitrogen) according to the manufacturer’s instructions. The assay is performed in a microplate read by a Mx3000P™ (Stratagene®) and extracted RNA was diluted to be used in reverse transcription (RT)-PCR assays.

Quantitative RT-PCR

The Affinityscript® QPCR cDNA Synthesis Kit (Agilent) was used for the ARN retrotranscription step on a Perkin Elmer 2400 thermocycler following the cycle conditions, 25°C for 5 min, 42°C for 15 min and 95°C for 5 min. Quantitative RT-PCR for cDNA amplification was performed with the Brilliant® II QPCR Kit (Agilent) as recommended by the manufacturer, under common cycle conditions (10 min at 95°C, 40 cycles of 30 s at 60°C on the Mx3000p thermal cycler (Stratagene). The primers were as follows: sens, 5'-CCC TGA ATG CGG CTA ATC-3' and reverse, 5'-ATT GTC ACC ATA AGC AGC-3'. The sequence of the probe was FAM-AAC CGA CTA CTT TGG GTG TCC GTG TTT-TAMRA. Enterovirus 71 RNA (Vircell) was used as standard for quantification.

Histological analysis

Tissues were fixed with Formol embedded with paraffin and then cut into 4 µm thick sections. The slices were deparaffinized twice in xylene for 10 min, then rehydrated through sequential concentrations of alcohol (100, 95, 70 and 50%) and finally with double distilled water. Afterward, slices were stained with hematoxylin for 15 min, washed in running water, differentiated with 1% HCL/ethanol and incubated in water for 5 min. For cytoplasm staining, slices were then immersed in 0.5 % eosin solution for 3 min and washed in water for 10 min. Sections were dehydrated again through sequential concentrations of alcohol (50, 70, 95 and 100°). Finally, slides were mounted and observed under Zeiss Axioscan (Z1) Scanner.

Serum level of glucose

Glucose level in serum of mice was measured with contour® XT from BAYER according to the manufacturer’s instructions. Two µl of serum were put on strips so that 0.6 µl was aspirated by capillarity. The results were expressed as mg/dl.

Statistical analysis

Graphs and analyses, Spearman test and t test when appropriate, were performed with GraphPad Prism V6.0 software, significance set at 0.05.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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