Prolonged Viral RNA Detection in Blood and Lymphoid Tissues from *Coxsackievirus B4 E2* Orally-Inoculated *Swiss* Mice

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Abstract: The spreading of viral RNA within *Swiss Albino* mice orally inoculated with *coxsackievirus B4 E2* strain (CVB4 E2) was studied by using RT-PCR and semi-nested-RT-PCR methods. Viral RNA was detected in various organs: pancreas, heart, small intestine, spleen, thymus, and blood at various post-infectious (p.i.) times ranging from 8 hr to 150 days. Our results show that (i) outbred mice can be infected with CVB4 E2 following an oral inoculation, which results in systemic spreading of viral RNA, (ii) CVB4 E2 infection can be associated with a prolonged detection of viral RNA in spleen, thymus and blood, up to 70 days p.i. and further in other organ tissues.

Enteroviruses (family: *Picornaviridae*) and especially *Coxsackievirus B* (CVB) have often been implicated in the etiology of type 1 diabetes mellitus. Enteroviral RNA has been found, by several investigators, in peripheral blood of 27 to 64% of patients with type 1 diabetes (12). In some cases, CVB RNA sequences showed a significant homology with CVB4 (6). In another study, sequences of CVB4 RNA homologous to *coxsackievirus B4 E2* (CVB4 E2) were found in peripheral blood mononuclear cells of patients with type 1 diabetes (20). CVB4 E2 is the best known diabetogenic enterovirus strain. It was isolated from the pancreas of a child who died from diabetic ketoacidosis and it was able to destroy β cells of the pancreas and to induce hyperglycemia in some susceptible mouse strains (21). A persistent infection of human pancreatic islet cells and thymic epithelial cells *in vitro* can be obtained with CVB4 E2 (3, 5).

The spreading of CVB4 E2 following its introduc-tion into the organism through a natural way has not been studied yet. Therefore, we developed an experi-mental model based on an oral inoculation of outbred *Swiss Albino* mice with CVB4 E2. The aim of the cur-rent study was to determine the targets of the infection and the kinetics of viral RNA in various tissues, particu-larly in lymphoid organs and blood.

Briefly, 3–4 weeks old *Swiss Albino* female mice (Pasteur Institute, Tunis, Tunisia), were orally inoculat-ed, by using a rigid canula, with $10^{4.74}$ TCID$_{50}$ of CVB4 E2 contained in 200 μl culture supernatant of infected-cells. CVB4 E2 (kindly provided by Dr. J.W. Yoon, Calgary, Canada) was propagated in Hep-2 cells (BioWhittaker) in Eagle's minimal essential medium (Gibco BRL) supplemented with 10% decomplemented fetal calf serum and 1% L-glutamine. Mice were treated according to general ethical rules and maintained under specific pathogen-free conditions with unlimited access to food and water. Two infected animals were sacri-ficed at different post-infectious (p.i.) times ranging from 1 hr to 180 days. Naïve mice served as negative

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controls. From each animal, blood was collected on EDTA and heart, thymus, pancreas, spleen, small intestine were removed, rinsed with PBS, snap-frozen in liquid nitrogen and stored at -80 C. Total RNA was extracted using the RNAgents Total RNA Isolation System kit (Promega) for blood samples, and using Tri-Reagent (Sigma) for other tissues, as described by Chomczynski and Sacchi, (7). Genome amplification was then performed using a single-tube method with the SuperScript™ One-Step RT-PCR with Platinum® Taq kit (Invitrogen), as previously described (10). Primer sense 006: 5'-TCCTCCGGCCCCTGAATGCG-3' and anti-sense 007: 5'-ATTGTCACCATAAGCAGC-CA-3' (Proligo) were selected within the 5' non-translated region of the enteroviral genome; generating a 155 bp fragment (22). Total RNA extracts from samples showing negative results by this method were submitted to a RT-PCR similar to the previous one except that the sense-primer 006 was replaced by an external one that we called 008 5'-GAGTATCAATAAGCTGCTTG-3' (Proligo) also located within the 5' non-translated region, generating a 414 bp fragment. A semi-nested-PCR was then performed on first amplification products, using 006 and 007 primers and the Platinum PCR SuperMix kit (Invitrogen) as recommended by the manufacturers. For each RNA sample, GAPDH mRNA was submitted to RT-PCR, using primer sense 5'-AAC-GACCCCTTCATTGAC-3' and anti-sense 5'-TCCAC-GACATACTCAGCAC-3' (Proligo), and used as a positive control to prove the absence of reaction inhibitors. The amplified products were analyzed by electrophoresis on a 2% agarose gel containing 0.5 mg ml^{-1} of ethidium bromide (Sigma) and visualized by using the Gel Doc 2000 system (Bio-Rad).

This is the first study describing the detection of viral RNA in CVB4 E2 orally-infected outbred mice. Table 1 gives all RT-PCR results and those of semi-nested-RT-PCR (sn-RT-PCR) that were positive. For control mice, all sampled tissues were negative (data not shown). For inoculated animals, CVB4 E2 RNA was found in all sampled tissue types at various p.i. times ranging from 8 hr to 150 days: from 16 hr up to 70 days in blood, thymus and heart, from 16 hr up to 90 days in pancreas, from 8 hr up to 70 days in spleen, and from 8 hr up to 150 days in small intestine. All samples, including those showing negative results for CVB4 E2 RNA, were positive for GAPDH mRNA, proving the RNA integrity and the absence of reaction inhibitors (results not shown). Our data show that a systemic spreading of CVB4 E2 following oral inoculation is possible and that outbred mice, in addition to inbred, diabetic, immuno-compromised or transgenic mice (4, 8, 9, 11, 18, 21), can be infected with that viral strain. The natural expression in the mouse of a coxsackievirus and adenovirus receptor strongly homologous to the human one (19), made possible the use of these animals as models to study the infection with CVB4 E2. We decided to use per-oral infected outbred mice in our experiments because such animals are much more representative of the natural variation in human population than inbred mice as underlined by other authors (1) and because it is important to consider the natural route of infection in experimental models.

CVB4 E2 strain was capable of targeting several organs, and its RNA was detectable up to 70 days and further (see Table 1). Our results concerning a prolonged viral RNA detection in heart, spleen, pancreas and small intestine are in agreement with those of other

Table 1. RT-PCR (and sn-RT-PCR) results on sampled tissues from CVB4 E2 orally-infected Swiss mice

Tissues	Time p. i.																					
	1 hr	2 hr	4 hr	8 hr	16 hr	24 hr	48 hr	72 hr	5 d	7 d	10 d	14 d	21 d	28 d	35 d	42 d	56 d	70 d	90 d	120 d	150 d	180 d
Blood	−−	−−	−−	−−	++	−−	−−	−−	−−	−−	+−	+−	−−/sn+	NS	NS	−−/sn+	−−	+−	−−	−−	−−	−−
Pancreas	−−	−−	−−	−−	++	+−	++	++	++	++	++	++	+−	++	++	−−	++	−−	−−/sn+	−−	−−	−−
Thymus	−−	−−	−−	−−	++	+−	++	++	++	++	+−	++	+−	++	++	+−	++	+−	−−	−−	−−	−−
Spleen	−−	−−	−−	+−	+−	+−	++	++	++	++	++	++	−−/2sn+	−−/2sn+	++	+−	++	+−	−−	−−	−−	−−
Heart	−−	−−	−−	−−	++	+−	++	++	++	++	+−	++	+−/sn+	++	++	+−/sn+	++	+−	−−	−−	−−	−−
Small intestine	−−	−−	−−	+−	++	+−	−−	+−	++	++	++	−−	+−	++	+−	−−	++	−−	−−	−−	−−/sn+	−−

p.i.: post-infection; hr: hour; d: day; NS: not sampled.
Two mice were tested at each time point (++: two mice showing positive results by RT-PCR; −−: two mice showing negative results by RT-PCR; +−: one mouse showing positive and another one showing negative result by RT-PCR).
sn-RT-PCR was performed only for samples showing negative results by RT-PCR. Only positive results by sn-RT-PCR are mentioned (sn+: one positive result by sn-RT-PCR, 2sn+: two positive results by sn-RT-PCR).

authors obtained in mice inoculated with CVB3 Nancy by the oral route (2, 10). The prolonged detection of viral RNA in the spleen is reminiscent of results of other investigations based on intraperitoneal inoculation by CVB3 Nancy (14, 16). The detection of viral RNA by sn-RT-PCR in pancreatic tissue was possible up to 90 days p.i. in our experiments with CVB4 E2 and up to 56 days p.i. in another study with CVB3 (2), which can be due to a stronger pancreatotropic property of CVB4 E2. Indeed, it has been reported that the pancreases of CD-1 mice intraperitoneally challenged with CVB4 E2, were still positive for viral RNA at 6 months p.i. (17).

Our results show that CVB4 E2 RNA can reach the thymus *in vivo* following an infection by a natural route and that, in these conditions, viral RNA can be found in thymus until 70 days p.i. (like in spleen and heart). In a previous investigation based on CVB3 intraperitoneal inoculation of SWR/J, *H-2q* mice, viral RNA in thymus was visualized by *in situ* hybridization, at a low level at day 6 but not at day 42 p.i. (14). In mice inoculated with CVB4 E2 through the intraperitoneal route, Chatterjee et al. (4) obtained a thymic infection; the virus was rapidly cleared from that organ but at 8 weeks p.i. a 150% increase in $CD4^-CD8^-$ thymic T cells was observed. It has been reported that a viral infection of thymus can facilitate the immune tolerance to antigens of that infectious agent, which can result in viral persistence (13, 15). In the human system, a prolonged detection of CVB4 E2 RNA has been obtained in thymic cells infected *in vitro*, which resulted in a sustained production of cytokines by these cells (3). In so far as CVB4 E2 RNA can be detected in thymus in experimental models, particularly after oral infection as demonstrated here, on one hand, and a CVB4 can disturb the function of that organ on the other hand, further studies are required to determine whether CVB4 infections in humans can modulate the thymic function and whether that can play a role in CVB4-induced diseases.

To the best of our knowledge, this is the first investigation reporting a prolonged detection of CVB RNA in mice blood. Our interest in the blood comes from the fact that enteroviral RNA of CVB subgroup members, especially CVB4, was detected in human blood from patients with type 1 diabetes with nucleotide sequence homologous to CVB4 E2 in some patients (6, 20). In an experimental model based on inbred male SWR mice inoculated with CVB3 by the intraperitoneal route (16), viral RNA was detected by quantitative RT-PCR in blood up to day 14 p.i. In the current study, CVB4 E2 RNA was detected from 16 hr up to 70 days p.i. in blood (see Table 1). However, CVB4 E2 RNA was not detected in every sample of blood or other tissues (see Table 1). For example, CVB4 E2 RNA was detected in

pancreatic samples at different p.i. times, from 16 hr up to 90 days, but neither at 42 days nor at 70 days. The discordance of viral RNA detection in blood and organs suggest that the positive RT-PCR tests for organs were not due to a contamination with viral RNA-containing blood and argue in favor of the presence of viral nucleotide sequences in the studied organ. Together, the variations of the detection of CVB4 E2 RNA in blood and other tissues in our experiments suggest that the circulation of enteroviral RNA within the organism can be different at various p.i. times. An alternative explanation is that our data reflect a different response to the infection due to natural individual variations within outbred mice.

In our experiments, viral RNA was detected as soon as 8 hr p.i. in spleen and small intestine, and 16 hr p.i. in the other tissue types. In the investigation conducted by Harrath et al. (10), per-oral infection of BALB/c mice with CVB3 Nancy affected the different organs in a more progressive way (the intestine at 2 hr, the heart at 1 day, the pancreas at 2 days and the spleen at 3 days p.i.). That difference can be attributed to distinct viral and mice strains. Our data suggest that CVB4 E2 can rapidly pass through the gut to reach the spleen and then spread towards various organs.

Our experimental model of CVB4 E2 infection by a natural route shows that viral RNA can be found by RT-PCR in blood and in immune system organs, spleen and thymus, up to 70 days p.i. In addition to these organs, CVB4 E2 RNA was detected in small intestine, heart and pancreas up to 56 days by RT-PCR and further by sn-RT-PCR. Together our data suggest that CVB4 E2 infection by a natural route can be associated with a prolonged detection of viral RNA within the organism, and that viral RNA can be detected in blood. Studies are in progress in our laboratory to determine whether the detection of enteroviral RNA in peripheral blood of patients with type 1 diabetes is related to a prolonged presence of enteroviruses which would be consistent with a persistent infection with these viruses in such patients.

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