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Relation between trace element levels in plasma and myocardium during coxsackievirus B3 myocarditis in the mouse

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Abstract

During most infections the plasma levels of trace elements change, but it is not clear if this reflects changes in the infected tissues. Coxsackievirus B3 (CB3) infection may result in viral replication, subsequent inflammation and changed trace element levels in the myocardium. In the present study, the trace element levels in the plasma and heart of adult male A/J mice were determined during the pre-inflammatory stage (day 4) of CB3 myocarditis for the following trace elements: aluminium (Al), arsenic (As), calcium (Ca), cobalt (Co), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), selenium (Se), silver (Ag), vanadium (V) and zinc (Zn). The severity of the infection was assessed through clinical signs of disease and trace element levels were measured through inductively-coupled plasma mass-spectrometry (ICP-MS). In the heart, the levels decreased for V (59%; $p < 0.01$), Co (38%; $p < 0.01$), Al (81%; $p < 0.01$), As (66%; $p < 0.01$) and Se (16%; $p < 0.01$). Increased levels were detected for Mn (13%; $p < 0.05$), Fe (48%; $p < 0.01$), Cu (34%; $p < 0.01$) and Ag (46%; $p < 0.01$). In the plasma, decreases were detected in the level of Zn (32%; $p < 0.05$), whereas increases were seen in Mn (362%; $p < 0.05$), Fe (272%; $p < 0.01$), Co (71%; $p < 0.05$), Cu (25%; n.s.) and Mg (43%; $p < 0.01$) levels. A correlation was found between the levels in plasma and myocardium for Co ($r_s = -0.636$; $p < 0.05$), Fe ($r_s = 0.764$; $p < 0.05$), Mn ($r_s = 0.682$; $p < 0.05$) and Mg ($r_s = -0.791$; $p < 0.05$). Thus, determination of some of these trace elements in the plasma may be useful to indicate target tissue involvement in the early pre- inflammatory stage of an infectious disease. Some of these elements are important nutrients for the immune system, while others may be associated with the development of disease complications, such as cardiac arrhythmias.

Abbreviations: CB3 – coxsackievirus type B3; p.f.u. – plaque-forming units; ICP-MS – inductively-coupled plasma mass-spectrometry.

Introduction

Characteristic host responses during early stages of acute infections include the synthesis of metal-binding proteins in the liver, such as metallothionein, ferritin and ceruloplasmin (Beisel 1998; Ilbäck *et al.* 1983; Sobocinski *et al.* 1978), and a flux of trace elements between blood and tissues (Beisel *et al.* 1974; Beisel 1998; Friman & Ilbäck 1998). Changed plasma levels of some trace elements have been used as markers of disease during experimental infections of varying etiology, including an increased ratio between plasma copper and zinc levels, and a decreased plasma iron concentration (Ilbäck *et al.* 1983; Beisel 1998; Percival 1998). However, little is known about the trace element flux between plasma and the body tissues and whether the observed changes in plasma reflect changes in the target tissues of the infectious microor-

ganisms. The trace element balance could have an influence on host defence mechanisms and also on the virulence of the infectious microorganism (Fraker *et al.* 1978; Cunningham-Rundles 1991; Beck *et al.* 1994; Beck & Levander 2000; Vartiainen *et al.* 1999).

Virtually all humans contract several enterovirus infections during their lifetime, but the majority pass without any apparent symptoms, or cause only minor illness of the upper respiratory or gastrointestinal tract. However, enteroviruses of the coxsackie B group may sometimes cause more severe disease such as myocarditis, pancreatitis or meningoencephalitis (Woodruff 1980). The pathogenesis of the murine infectious model of coxsackie B virus type 3 (CB3) closely resembles that of CB3 myocarditis in humans and is characterized by a short lasting viremic phase, during which viruses infect the target cells, primarily in the heart and pancreas (Woodruff 1980). Already at this stage, before inflammatory lesions are histologically evident, the myocardial levels of several trace elements are affected (Funseth *et al.* 2000). Specific neutralizing antibodies and cellular immune responses then destruct the virus infected cells leaving areas of inflammatory lesions and necrosis, finally leading to scar formation (Chow *et al.* 1991; Godeny & Gauntt 1987; Henke *et al.* 1995; Huber *et al.* 1993).

In the present study, the trace element levels in plasma and myocardium were studied simultaneously during early CB3 infection using a well-described murine myocarditis model of the human CB3 virus infection. The intention was to identify early trace element changes in the plasma, and whether they are related to the trace element changes in the myocardial tissue that we have found previously in this target organ of the CB3 virus (Funseth *et al.* 2000). If so, plasma determination of trace elements might be a potentially useful tool to indicate the extent of developing target tissue involvement in the infectious process and, possibly, to monitor the response to therapy when available.

Materials and methods

Mice

Adult male A/J-mice from Charles River were maintained at the Animal Department, Biomedical Centre, Uppsala, Sweden. The mice were randomly assigned to groups of similar initial mean body weight and housed individually at 23 ± 1 °C on a 12 h light/dark cycle behind hygienic barriers with free access to food (R3; Ewos, Södertälje, Sweden) and water. Control and infected mice were studied simultaneously.

Virus

A myocarditic coxsackievirus type B3 (CB3) was used (Woodruff & Kilbourne 1970). Virus was propagated in HeLa cells, which were grown in Eagles' minimal essential medium supplemented with 5% fetal calf serum and antibiotics. Virus titres were determined on HeLa cells as plaque-forming units (p.f.u.) and a stock solution was stored at −20 °C until use. The stock solution of 10^7 to 10^8 p.f.u. ml^{-1} was diluted with phosphate buffered saline to get 10^5 p.f.u. ml^{-1}.

Infection, tissue sampling and tissue preparation

On day 0 of the infection, each mouse was inoculated i.p. with approximately 2×10^4 p.f.u. of CB3 virus. This dose and route of administration had in prestudies of adult male A/J mice been shown to produce 30% lethality from 7–9 days after inoculation. Uninfected controls were sham-inoculated with HeLa cell medium.

Infected mice were anaesthetised with Hypnorm/Dormicum and sacrificed on day 4. Sham-inoculated mice were sacrificed to serve as healthy controls. Body and organ weights for pancreas, liver, kidney and heart were recorded. Samples of ventricular myocardium and plasma for analysis of trace element concentrations were frozen at −70 °C.

Determination of trace elements

To assess the trace elements aluminium (Al), arsenic (As), calcium (Ca), cobalt (Co), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), selenium (Se), silver (Ag), vanadium (V) and zinc (Zn), the tissue samples were decomposed using ultra-pure nitric acid (Scan Pure; Chem Scand AS, Elverum, Norway) in a steel bomb (MeAna-Konsult; Uppsala, Sweden). Tissue and plasma samples of about 0.1–0.3 g were weighed and put in quartz tubes. One ml of 65% nitric acid/0.1 g sample dry weight was added and the tubes were sealed with a teflon lid and put into steel bombs which were sealed with exactly the same momentum. The bombs were then heated in an oven to 180 °C for 4 h. After decomposition, an internal standard (indium) was added and the samples diluted in 10 ml of high purity water from an ElgaStat UHP (Elga Ltd.; High Wycomb, Buckshire, England). The

Table 1. Body and organ weights of uninfected male A/J control mice ($n = 6$) and of CB3-infected mice on day 4 of infection ($n = 5$)

Organ weight	Control	CB3 day 4
Body weight (g)	28.5 (7.6, 11)	27.8 (4.1, 5.4)
Heart weight (mg)	116 (14, 44)	116 (13, 35)
Pancreas weight (mg)	113 (26, 28)	86.0 (130, 160)
Liver weight (mg)	1150 (150, 400)	1090 (73, 150)
Kidney weight (mg)	211 (84, 97)	190 (30, 51)

Data expressed as median (interquartile range, total range). Significant differences between the controls and the infected mice are indicated as $^{*}p < 0.05$ and $^{**}p < 0.01$.

water quality was maintained at more than 18 Mωcm. All handling of the samples was done in a clean room. The trace element content of the samples was then measured by inductively-coupled plasma mass-spectrometry (ICP-MS; Perkin-Elmer SCIEX ELAN 6000; Perkin Elmer Corp.; Norwalk, CT, U.S.A.). For quality control, every fifth sample was a Certified Reference Material of Bovine muscle (Community Bureau of Reference, Brussels, Belgium), resulting in an overall precision of less than 5% and an overall accuracy of less than 8%.

Statistical analyses

Statistical analyses when comparing results in infected and sham-inoculated control mice were performed in StatView 4.5 using the nonparametric Mann-Whitney U test. For correlations the Spearman's rank test was used.

Results

The infection caused a cumulative mortality of 15% on day 4, corresponding to the peak of viremia. At this point virtually no inflammatory cells have yet accumulated in the myocardium in this infection model (Huber 1993). There was no significant loss of body or organ weights as a result of the disease (Table 1).

On day 4 of the infection, the myocardial levels (Table 2) decreased for Al (81%; $p < 0.01$), As (66%; $p < 0.01$), Co (38%; $p < 0.01$), Se (16%; $p < 0.01$) and V (59%; $p < 0.01$). Increases were detected in the levels of Ag (46%; $p < 0.01$), Cu (34%; $p < 0.01$), Fe (48%; $p < 0.01$) and Mn (13%; $p < 0.05$). No significant changes in the myocardial levels of Mg, Ca and Zn were detected at this stage of the disease.

On day 4 of the disease, the plasma levels (Table 3) of several trace elements were also changed. The Zn level was decreased by 32% ($p < 0.01$), whereas increases were detected in Mn (362%; $p < 0.05$), Fe (272%; $p < 0.01$), Co (71%; $p < 0.05$), Cu (25%; n.s.) and Mg (43%; $p < 0.01$) levels. As expected, the Cu/Zn ratio increased by 85% ($p < 0.01$). No significant changes were observed in the plasma for Ag, Al, As, Ca, Cu, Se or V at this stage.

Analysis of infected and uninfected mice together revealed a significant negative correlation (Table 4) between the plasma and myocardial levels for Co ($r_s = -0.636$; $p < 0.05$) and Mg ($r_s = -0.791$; $p < 0.05$), whereas a positive correlation was observed for Fe ($r_s = 0.764$; $p < 0.05$) and Mn ($r_s = 0.682$; $p < 0.05$).

Discussion

During the present infection, the levels of most of the studied trace elements changed both in the plasma and in the heart. Significant correlations between levels in plasma and heart were found for a few. Thus, negative correlations were observed for Co and Mg, whereas positive correlations were found for Fe and Mn. In accordance with previous experimental studies of various infectious diseases, the Cu/Zn ratio in plasma increased markedly, reflecting a decrease in plasma Zn and an increase in plasma Cu, whereas, somewhat unexpectedly, Zn in the heart tissue did not change significantly (Beisel *et al.* 1974; Ilbäck *et al.* 1983). Longitudinal experimental studies of the elements for which significant correlations between plasma and tissue were found are needed to evaluate the potential of their plasma levels as indicators of events in the target tissue.

Depending on the specific target organ of an infection, the local concentration of specific elements may be important. It is well-known that during the course of inflammatory heart disease, the inflammatory lesions, necrosis, fibrosis and subsequent calcification sometimes disturb the electric conduction, causing arrhythmias and occasionally sudden death. However, we have previously observed sudden deaths during exercise also in experimental infections without myocardial inflammation, suggesting additional causative factors (Friman *et al.* 1982; Ilbäck *et al.* 1984). Mg-deficiency has been associated with cardiac arrhythmias and sudden death, and in the clinical setting intravenous administration of Mg is effective for

Table 2. Concentration (μg g^{-1} wet tissue wt.) of selected trace elements in the myocardium of uninfected male A/J control mice ($n = 6$) and of CB3-infected mice ($n = 5$) on day 4 of the infection

Trace element	Trace element concentration in controls (μg g^{-1})	Trace element concentration in CB3 infected mice (μg g^{-1}), day 4 of the infection
Aluminium (Al)	1460 (140, 190)	278 (130, 150)**
Arsenic (As)	15.1 (6.7, 8.5)	5.10 (5.0, 5.7)**
Calcium (Ca)	44770 (11586, 14811)	41042 (23000, 30000)
Cobalt (Co)	56.3 (0.6, 2.2)	34.9 (1.4, 2.1)**
Copper (Cu)	6700 (346, 867)	8950 (550, 1200)**
Iron (Fe)	64600 (2700, 5900)	95700 (9800, 16000)**
Magnesium (Mg)	257734 (21987, 28764)	267408 (32618, 71442)
Manganese (Mn)	753 (75, 220)	851 (54, 84)*
Selenium (Se)	346 (20, 100)	291 (14, 31)**
Silver (Ag)	5.40 (1.1, 1.6)	7.51 (1.5, 2.1)**
Vanadium (V)	9.10 (2.8, 7.8)	3.70 (0.5, 1.1)**
Zinc (Zn)	31400 (3000, 7200)	35100 (5600, 9700)

Data expressed as median (interquartile range, total range). Significant differences between the controls and the infected mice are indicated as $^{*}p < 0.05$ and $^{**}p < 0.01$.

Table 3. Concentration (μg ml^{-1}) of selected trace elements in plasma of uninfected male A/J control mice ($n = 6$) and of CB3-infected mice on day 4 of the infection ($n = 5$)

Trace element	Trace element concentration in controls (μg ml^{-1})	Trace element concentration in CB3 infected mice (μg ml^{-1}),
Aluminium (Al)	92.9 (19, 42)	122 (81, 120)
Arsenic (As)	17.0 (2.5, 6.3)	16.3 (3.8, 4.7)
Calcium (Ca)	82800 (4600, 21000)	94595 (7304, 15019)
Cobalt (Co)	1.07 (0.3, 1.6)	1.82 (0.5, 1.1)*
Copper (Cu)	798 (340, 400)	1000 (560, 950)
Iron (Fe)	3780 (920, 2700)	10300 (7700, 12000)**
Magnesium (Mg)	17000 (2100; 9500)	24300 (12000, 14000)**
Manganese (Mn)	5.39 (0.9, 3.3)	19.5 (31, 47)*
Selenium (Se)	325 (26, 88)	328 (44, 133)
Silver (Ag)	0.19 (0.1, 0.1)	0.25 (0.1, 0.2)
Vanadium (V)	3.63 (0.8, 1.0)	4.07 (0.3, 0.5)
Zinc (Zn)	1120 (160, 360)	760 (93.5, 155)**
Cu/Zn-ratio	0.71 (0.33, 0.48)	1.32 (0.58, 0.98)**

Data expressed as median (interquartile range, total range). Significant differences between the controls and the infected mice are indicated as $^{*}p < 0.05$ and $^{**}\ p < 0.01$.

Table 4. Correlation between myocardial (μg g^{-1}) and plasma (μg ml^{-1}) trace element concentrations in uninfected control mice and in infected mice on day 4 of infection

Trace element	r^s
Aluminium (Al)	−0.300
Arsenic (As)	0.009
Calcium (Ca)	0.327
Cobalt (Co)	−0.636*
Copper (Cu)	0.282
Iron (Fe)	0.764*
Magnesium (Mg)	−0.791*
Manganese (Mn)	0.682*
Selenium (Se)	0.291
Silver (Ag)	0.556
Vanadium (V)	−0.580
Zinc (Zn)	−0.382

The Spearman rank correlation coefficient (r_s) was considered significant at $p < 0.05$ (indicated as *).

suppression of ventricular ectopic foci, e.g. to overcome torsade de pointe-arrhythmias (Altura & Altura 1991; Swain & Kaplan-Machlis 1999). Even though the present study of early infection showed no significant changes in the Mg levels in plasma or heart, a significant negative correlation was found. In an earlier study of sequential trace element changes in the myocardium, Mg levels did not decrease until inflammatory lesions are present (Funseth *et al.* 2000). Thus, repeated sampling of plasma Mg levels would be warranted to detect changes predisposing for harmful arrhythmias.

The total myocardial level of Ca was not significantly different from that in healthy control mice at this early stage of the infection, i.e. before histologically evident inflammatory lesions appear, even if we have previously found Ca-accumulation in both inflamed and noninflamed areas during the subsequent phase of acute viral myocarditis (Ilbäck *et al.* 1989, 2001). Nevertheless, Ca has been shown to play an important role at the early stage of cardiomyocyte apoptosis induced by the CB3 virus (Li *et al.* 1999). Furthermore, calcification at later stages of the disease is associated with a worsened prognosis in viral myocarditis in humans, as well as in mice (Chow *et al.* 1991; Stallion *et al.* 1994).

Nutritional imbalance has been suggested as a factor contributing to the emergence of new infections (Morse 1997). Ex vivo studies report that increased Mn levels, as compared to the normal in vivo levels, are associated with an increased virulence of human immunodeficiency virus type 1 (Vartiainen *et al.* 1999). In experimental studies, Se-deficiency in mice seems to drive the mutation of CB3 into more virulent variants, causing more severe disease (Beck *et al.* 1994; Beck & Levander 2000). Conversely, Se-supplementation is immune stimulatory and is associated with milder disease even when given to the initially Se-nondeficient host (Ilbäck *et al.* 1989). The importance of a normal Se balance in human myocardial disease has been widely discussed concerning Keshan-disease, a cardiomyopathy seen in certain areas of China where the Se-intake in the general population is low and coxsackievirus infection has been serologically verified (Bo-Qi 1983). The sequential change in myocardial Se levels during the development of myocarditis seems to be biphasic, i.e. at the peak of viremia at day 4 there is a decrease, turning into an increase on day 7, the latter probably representing a compensatory effect (Funseth *et al.* 2000). Accordingly, in the present study, there was a myocardial decrease in Se at day 4 but no change in plasma, nor any correlation between plasma and tissue levels.

There is no explanation for the changed levels of V, Co, Al, Ag and As in the heart, nor for Co in the plasma during infection. These trace elements have no known function in host defence reactions to invading microorganisms. However, it cannot be excluded that they may compete or interact with other trace elements or metals (Ilbäck *et al.* 2000; Wicklund-Glynn *et al.* 1998). For other diseases it has been emphasized that combinations of trace element changes rather than an altered level of a single element may be disease-promoting (Fields 1999). In ischaemic heart disease, Cu affects myocardial contractility, whereas the level of Mg, Ca, Cu and Zn are closely related to cardiac rhythm disturbances (Barandier *et al.* 1999).

Zn is intimately involved in the regulation of immune function, and consistent sequential alterations in the metabolism of Zn, as well as of Cu, occur during immune activation and infectious processes (Beisel *et al.* 1974; Driessen *et al.* 1995; Fernandes *et al.* 1979). It has also been found that Zn localizes to actively healing wounds, reaching maximal concentrations within 24–48 h after injury (Savlov *et al.* 1962). These characteristic alterations in trace element metabolism are part of the acute phase reaction

following infection and trauma (Beisel 1998) and are essential for optimal metabolic processes and immune responses. Determination of Zn and Cu have been used for early detection and monitoring of experimental infectious disease (Beisel *et al.* 1974; Ilbäck *et al.* 1983). Accordingly, an increased Cu/Zn-ratio was observed in plasma in the present infection and, also, an associated increase in the Cu level in the myocardium. Cu, as well as Se, is an important element in antioxidative enzymes (Beisel *et al.* 1974; Barandier *et al.* 1999).

An increased plasma level of Fe is well-documented in infectious diseases, in general occurring concomitant with the induction of the Fe-binding protein ferritin in the liver (Beisel *et al.* 1974). In the present study, both plasma and myocardial levels of Fe increased and were significantly correlated. An increased Fe level in the myocardium even before lesions become evident might also reflect an initiation of tissue damage caused by free radical formation. Hiraoka *et al.* (1993) indicated that the superoxide anion could be responsible for myocyte injury in CB3 myocarditis in mice. If part of the Fe increase consists of ionised Fe, then the Fenton-Haber-Weiss reaction would produce superoxide anions. Thus, the plasma levels seemed to reflect myocardial changes and deserves further evaluation in longitudinal studies as a potential marker of tissue involvement.

In conclusion, it has been suggested that the detection and correction of trace element imbalances could diminish individual risk factors for various diseases, including coronary heart disease, coxsackievirus infections and the common cold (Fields 1999; Ilbäck *et al.* 2000; Prasad *et al.* 2000). In the present study, important markers of disease were the plasma changes in Cu, Zn, Fe and Mg and the myocardial tissue changes in Se, Fe and Cu. The levels of Fe, Mg, Mn and Co in plasma and myocardium were significantly correlated. Among those, Fe and Mg are known to be important elements in the metabolism of the myocardium and immune system. Increased plasma levels of Fe indicates tissue involvement in the disease process, whereas plasma Mg was unchanged at this early stage of the infection, although tissue levels were increased. Thus, changes in the plasma level of some of these trace elements, in addition to traditional markers such as C-reactive protein and organ-specific enzymes, may be potentially useful for monitoring infectious diseases involving various target tissues. Some of these elements are important nutrients for the immune system, while others may be associated with the development of disease complications, such as cardiac arrhythmias. These results thus warrant further evaluation in longitudinal studies.

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