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Arsenic Influences Virus Replication in Experimental Coxsackievirus B3 Infection

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Abstract
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Trace elements are essential for the host defence against infections, and during common infections, the balance of trace elements is changed in serum and tissues. Supplementation with selenium (Se), an essential trace element, is known to decrease the severity of coxsackievirus B3 (CVB3) infection in mice. Even the non-essential trace element arsenic (As) seems to influence the replication of some viruses.

During the course of an acute CVB3 infection in mice, Se concentrations decreased in most tissues and were negatively correlated to viral load in our study. However, As concomitantly decreased in most tissues. As has previously been shown to interfere with the balance of essential trace elements. However, in the present study As supplementation in healthy mice resulted in minor effects on seven studied trace elements in serum and tissues. The effects of As supplementation were more pronounced in CVB3-infected mice, with an increase in As, but a decrease in Se in most tissues when compared with non-infected mice.

As supplementation during CVB3 infection in mice decreased viral RNA concentrations in the brain (97%) and pancreas (75%), two of the target organs of this infection. *In vitro* experiments indicate that As caused an impaired virion assembly or release. *In vivo*, infection-induced expression of the host defence-associated genes nuclear factor κB (NFκB) and interferon γ (IFN-γ) were unaffected by As supplementation, except for an earlier increase in IFN-γ in the brain.

In conclusion, a clinically relevant dose of As decreased the replication of CVB3 *in vitro* and *in vivo*. This antiviral effect *in vivo* was not related to changes in specific trace elements or in the host's immune-mediated defence. Although the mechanism underlying the observed effect on viral replication remains to be further elucidated, As seems to be an intriguing trace element to study in the pursuit of new antiviral drugs.

Keywords: coxsackievirus B3, trace elements, arsenic, selenium, virology, IFN-γ, NFκB

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Molin, Y**., Frisk, P., Ilbäck, NG. (2009) Viral RNA kinetics is associated with changes in trace elements in target organs of Coxsackievirus B3 infection. *Microbes and Infection*, 11(4):493-499.

II **Molin, Y**., Frisk, P., Ilbäck, N.G. (2008) Sequential effects of daily arsenic trioxide treatment on essential and non-essential trace elements in tissues in mice. *Anti-cancer Drugs*, 19(8): 812-818.

III **Molin, Y**., Frisk, P., Ilbäck, NG. (2009) Arsenic trioxide affects the trace element balance in tissues in infected and healthy mice differently. *Anticancer Research*, 29(1):83-90.

IV **Molin, Y**., Frisk, P., Hjelm, E., Blomberg, J., Friman, G., Ilbäck, N-G. Arsenic trioxide influences viral replication in target organs of coxsackievirus B3-infected mice. (Manuscript)

V **Molin, Y**., Gadhasson, I., Frisk, P., Ilbäck, N-G. Are antiviral effects of arsenic trioxide in coxsackievirus B3 infection associated with NFκB and IFN-γ expression in infected mouse tissues? (Manuscript)

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Cover picture of coxsackievirus B3 with the courtesy of Dr. Jean-Yves Sgro, University of Wisconsin-Madison. PDB_ID: 1COV. *Muckelbauer, J.K. et al. Structure determination of coxsackievirus B3 to 3.5 A resolution. (1995) Acta Crystallogr.,Sect.D 51: 871-887.*

Contents

Abbreviations

APL	Acute promyelocytic leukaemia
As	Arsenic
As_2O_3	Arsenic trioxide
AS3MT	Arsenic (III) methyltransferase
BBB	Blood-brain barrier
Cd	Cadmium
CPE	Cytopathogenic effects
Cq	Quantification cycle
Cu	Copper
CVB3	Coxsackievirus B3
DCM	Dilated cardiomyopathy
EV	Enterovirus
Fe	Iron
GSH	Glutathione
HEV	Human enterovirus
Hg	Mercury
HSV-1	Herpes Simplex-virus 1
ICP-MS	Inductively coupled plasma mass spectrometry
IFN	Interferon
Mg	Magnesium
NF-κB	Nuclear factor κB
Pfu	Plaque-forming unit
RT-PCR	Reverse transcriptase polymerase chain reaction
Se	Selenium
Zn	Zinc

Introduction

During the initial phase of an infection, the activation of the immune system of the infected host causes an increased need for energy and nutrients (14). This increased need is likely also true for the invading microorganisms, all resulting in an increased metabolic rate in the host (80). The acute-phase reaction of an infection includes a great number of events, all of which serve to destroy the invading microorganism and restore tissue functions, i.e. fever, hormonal changes, immune cell activation, increased synthesis and release of cytokines, antibody production and complement activation and a simultaneous flow of trace elements between blood and tissues (14, 80). The proinflammatory cytokines, secreted by different immune cells as a response to an infection, stimulate the synthesis of metal-binding proteins and liver uptake of trace elements and amino acids, where they are needed for the synthesis of acute-phase proteins (14). In fact, about 30% of all mammalian proteins are metalloproteins, with one or more metals in the active centre (95). Therefore, tissues such as the liver that produce acute-phase proteins have an increased need of trace elements during the initial steps of an infection. Consequently, the balance of several trace elements in the body is changed during most infections (14, 132).

In both eukaryotic and prokaryotic cells trace elements, such as selenium (Se), zinc (Zn), copper (Cu) and iron (Fe) are essential for metabolic processes (26, 132). Trace elements act as part of coenzymes involved in DNA replication, protein synthesis, immune function and antioxidative processes (26, 132). Trace elements are thus crucial for the host defence and a redistribution of these elements between body compartments occurs during infections (38, 52, 73, 74). The risk for adverse health effects during an infection may increase with a disturbance in the balance of trace elements, especially with those having effects directed to the immune system (9, 10, 80). Based on previous studies, it is reasonable to assume that restoration of the disturbed balance, i.e. supplementation or chelation of specific trace elements may speed up the recuperation of an infectious disease.

There is no satisfactory antiviral treatment for enterovirus infections and trace elements may have a new potential in the field of antiviral therapy. Both essential trace elements (e.g., Se and Zn) and non-essential trace elements (e.g., arsenic, As) have been found to inhibit viral replication *in vitro* (88, 97, 101), and of these, As is the least studied trace element in this context. Thus, it is vitally necessary to investigate both disease-protecting and

disease-promoting effects of different essential and non-essential trace elements. The present series of studies was designed to investigate the interactions between trace elements and infections and, more specifically, whether As has the potential to be used in antiviral treatment.

Physiological role of trace elements

The term trace element means that the element is present in biological fluids in concentrations of less than 100 µg/g wet weight (1). An essential trace element is needed in small quantities for growth and development of an organism. The trace elements currently essential for humans are the metals Cu, chromium (Cr), Fe, manganese (Mn), molybdenum (Mo) and Zn, the metalloid Se and the non-metal iodine (I) (66). There is evidence that other trace elements, including fluorine (F), boron (Bo), cobalt (Co) and vanadium (V) may also have vital physiological functions in humans (66).

Non-essential trace elements, such as As, cadmium (Cd) and mercury (Hg), when present in humans at trace levels, may also interact metabolically with nutritionally essential trace elements (54, 62). These interactions may partly be explained by the induction of metal-binding proteins (e.g., metallothioneins) that are ubiquitously expressed in almost all mammalian tissues (62, 129). Synthesis of these metallothioneins can be induced by Cd, Zn, Cu, Hg, As and gold, i.e. both essential and non-essential trace elements (22, 100, 112). Accordingly, essential and non-essential trace elements may compete with each other in metal-binding/transporting proteins, which may result in a changed blood and tissue balance of the trace elements (80).

Trace elements and infection

There are several trace elements essential for the host defence against infections. Zn, Cu, Fe and Se are all trace elements required for a properly functioning immune system, with effects not only directed to immune cells but also on antioxidant status and inflammation (26). Deficiencies in these essential trace elements can therefore suppress several components of both humoral and cell-mediated immune responses (3, 7, 149). However, the importance of other essential trace elements in the immune system has not been thoroughly investigated.

During many acute infections, there is a rapid flux of Fe from the plasma into the liver, where Fe may bind to ferritin and is not released until the infection has resolved (14, 132). Moreover, Zn is sequestered from serum to the liver during several infectious diseases. Conversely, the plasma concentration of Cu is increased during generalised infections, probably because of an increased synthesis and release of the Cu-binding ceruloplasmin from the

liver. During infection, this results in an increased plasma Cu and Zn ratio, referred to as part of the acute-phase reaction (14, 132).

The concentrations of trace elements in tissues are also changed during both bacterial and viral infections, mainly in the target organs of the specific infection (13, 38, 74, 80, 84). Furthermore, trace elements are distributed quantitatively differently to tissues during an infection (76, 79). Moreover, an infection can change the gastrointestinal uptake of trace elements (both essential and non-essential) (83). These changes in trace element balance may be explained by an increased synthesis of metal-transporting/binding proteins that occur in serum and tissues during infections (22, 53, 84). In coxsackievirus B3 (CVB3) and other infections, levels of metallothioneins, or other metal-binding/transporting proteins, such as Zn-T5, divalent metal transporter 1 (DMT-1) and hepcidin, are induced in serum and target organs of the infection, which is considered part of the acute-phase reaction (22, 37, 51, 82, 83). It is believed that these events occur in order to provide trace elements for synthesis of acute-phase proteins in the liver and other tissues, as well as to change the serum balance of trace elements to obstruct microbial growth/replication (14, 132). The increase in metal transporters/binders may also result in an increased tissue uptake of non-essential trace elements resulting in potentially toxic concentrations in these tissues (84). Subsequently, during an infection, the tissue distribution of non-essential trace elements to which the individual is being exposed may be different when compared with healthy individuals (76, 79). Another possible outcome of this induction and production of metal transporters is that a deficiency of essential trace elements may develop in infected tissues (84). As a consequence, the interaction between infection and nutrients may not only influence the outcome of the infection but may also influence the toxicity of trace elements and thereby increase the possibility for detrimental effects, even at exposure levels normally regarded as safe (81).

Trace elements and virulence

Essential trace elements are not only important for the immune system and the host defence but may also be important for replication and virulence of microorganisms. Evidence indicates that Se-dependent glutathione peroxidase modules are encoded in a number of RNA viruses, including potentially serious human pathogens (e.g., HIV-1, hepatitis C virus, CVB3 and measles virus) (170). Moreover, Zn-containing proteins seem to be essential for efficient reverse transcription of HIV-1 (23). Furthermore, it is evident that most bacteria need Fe for optimal growth (116, 132). A changed balance of essential or non-essential trace elements might therefore affect both the host immune system and replication/virulence of the invading pathogen and affect the outcome of an infection.

Essential trace elements

Selenium

Se, which is involved in the functioning of macrophages and T-lymphocytes, is an essential component of the antioxidant enzyme glutathione peroxidase (102, 168).

Benign strains of CVB3 and influenza A (H3N2) were found to cause more severe myocarditic lesions in Se-deficient mice (8, 10). The effect seen in CVB3 was attributed to mutations in the viral genome that made the amyocarditic strain genetically more similar to a myocarditic strain (12). The influenza infection during concomitant Se deficiency resulted in significant alterations in mRNA levels of cytokines and chemokines involved in pro-inflammatory responses (10). These findings contrast to the observation of less pronounced heart damage [as caused by herpes-simplex virus 1 (HSV-1)] in Se-deficient mice compared with Se-adequate mice (61). Such findings show that there are different preferences of trace elements for different microorganisms.

Se deficiency is also associated with Keshan disease, i.e. inflammation and degeneration of cardiac muscle tissue, where a concomitant CVB infection has been discussed as a possible contributing factor (103).

Se supplementation, on the other hand, has been shown to have protective effects on the development of inflammatory lesions that occurs in CVB3-induced myocarditis (86). Furthermore, a Se-containing protein was found to reduce HIV-1 transcription *in vitro* (88). Although beneficial effects of Se supplementation in critically ill patients have sporadically been reported, it has not been sufficiently evaluated in the clinical setting (166).

Zinc

Zn, which is needed for DNA synthesis, apoptosis and T-cell maturation, is found in almost every human cell (26). Accordingly, acute Zn deficiency was found to decrease survival time after pneumococcal infection in mice (153). It has also been suggested that Zn may increase responses of Interferon (IFN) α therapy in hepatitis C patients (126). However, the level of Zn supplementation appears crucial because both excessive and deficient amounts of Zn have been linked to reduced survival in HIV-1-infected individuals (6).

Zn has also been investigated as a possible therapeutic agent against “the common cold” caused by rhinoviruses, but without consistent results (60, 113, 159).

Copper

Cu is a cofactor for ceruloplasmin, a protein involved in iron metabolism. The physiological role of Cu seems to be mediated through the Cu-containing metalloenzymes (66).

Bacterial growth has been shown to be inhibited in the presence of elevated Cu concentrations and this may thus be used to conquer the bacterial infection (132). Moreover, Cu deficiency in mice was found to increase the cardiac pathology of both myocarditic and amyocarditic strains of CVB3, possibly as a result of a decrease in circulating CVB3-specific IgG2a antibodies (149).

Iron

Fe is included in the heme complex as an essential component of the cytochrome proteins, i.e. in the oxygen-transporting proteins in erythrocytes. Fe is also believed to be involved in T-cell immunity (66).

Fe has been shown *in vivo* to positively affect the growth of several bacteria (121). Thus, increasing evidence supports the theory that the decrease in Fe concentrations seen in blood during infection is part of the strategy of the immune system to obstruct bacterial growth (132), although these changes in serum do not always reflect the changes in infected tissues (38). Furthermore, excess amounts of Fe during a concomitant deficiency in vitamin E seem to increase CVB3 titres and heart muscle damage in mice (11).

Non-essential trace elements

Both essential and non-essential trace elements change in serum and tissues during the course of infections. Benyamin et al showed that As decreased in target organs (i.e. the heart, plasma and pancreas) of CVB3-infected mice, although it remained unchanged in brain (15). In addition, the tissue distribution of Ni, Cd and Hg has been shown to be changed during CVB3 infection in mice (50, 76, 79). In addition, these trace elements were found to affect the immune response (78, 85). Exposure to Ni reduced the activity of NK cells during CVB3 infection in mice (75), whereas the number of B cells in CVB3-induced myocarditic lesions was reduced as a consequence of Cd exposure (78). Moreover, Cd has been shown to negatively affect immune function and to increase the severity of symptoms and mortality during several different viral infections in mice (33, 146, 157).

Arsenic

We are constantly exposed to As from natural sources, such as soils, water and air (122). Based on epidemiological studies and several experimental studies in animals, As is classified as a carcinogen in humans (2). Furthermore, As poisoning is a major health issue in India, Bangladesh and several south American countries (137). The maximum drinking water level recommended by WHO is 10 µg/l (2).

These negative effects contrast to the fact that different As compounds have been used for medical purposes for more than 2000 years (36). In fact,

it has even been proposed that As might be an essential trace element with a physiological role (160).

Metabolism of As

The liver is the most important site of As methylation, although most tissues show As methylating activity (161). In most mammals the end metabolites of As are methylarsonic acid (MMA) and dimethylarsinic acid (DMA) (Figure 1), which are less tissue-reactive than inorganic As (161). Thus, the metabolism of As was thought to be a detoxification step, although it has been shown that these end metabolites possibly are more cytotoxic, genotoxic and more potent enzyme inhibitors than the inorganic As (155). Furthermore, there are differences in excretion between species, such as in mice where more than 99% of an oral dose of DMA was eliminated from the body within 3 days, contrasting to about 50% in rats during the same time period (162). Urine is the main excretion route for arsenicals (134).

The primary biochemical mechanism of As toxicity is binding of the element to cellular thiol groups, resulting in inhibition of numerous cellular enzyme systems (68, 152). The intracellular thiol glutathione (GSH) seems to be involved in methylation of As (161), and consequently, the sensitivity of various tumour cell lines to As-induced apoptosis was inversely related to intracellular GSH concentrations (32). Furthermore, a methyltransferase that catalyses these methylation reactions has been identified in humans, i.e. arsenic (III) methyltransferase (AS3MT) (106). Genetic variances in this gene have been associated with inter-individual differences in As methylation (67, 143). In addition, a significantly higher As methylation in women in childbearing age has been reported, indicating a hormonal effect on As methylation (107). Thus, there are significant inter-individual variations in As metabolism and the risk of toxicity seems to be different between individuals.

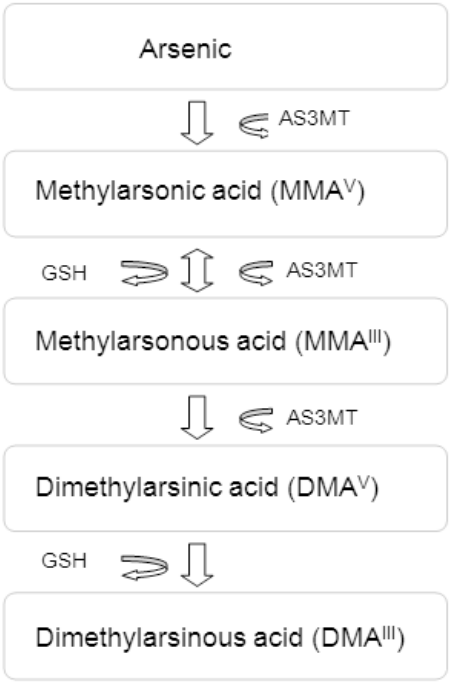

Figure 1. A schematic picture of the assumed arsenic metabolic pathway, although little is known about the *in vivo* reduction of DMA^{v} to DMA^{III} (58). GSH: glutathione, AS3MT: arsenic (III) methyltransferase.

Another possible intracellular effector molecule of arsenicals is the transcription factor nuclear factor κB (NFκB). Low levels of As were shown to increase the activity of NFκB (5), whereas higher concentrations of arsenicals have been shown to inhibit NFκB activity and subsequently induce apoptosis (114, 141). Thus, the effects on NFκB may be dependent on concentration of the arsenical, and possibly also the cell type used (35).

Tissues with high concentrations of thiols (e.g., keratin rich tissues such as skin, hair and nails) show the longest retention time and the highest concentrations of As (108, 134).

Arsenic used in medical therapy

Arsenicals have been used as medical therapy for over 2000 years (36). In the 1700s, Fowler's solution became a standard treatment for various diseases, (e.g., anaemia, leukaemia, asthma and psoriasis) (163), and 200 years later, in the early 1900s, Salvarsan was introduced. It was used to treat syphilis and African trypanosomiasis and soon became one of the most prescribed drugs in the world (36).

Today, arsenic trioxide (As_2O_3) is considered a relatively safe and effective treatment for relapsing acute promyelocytic leukaemia (APL) (36). Currently, several clinical trials are investigating the effect of As_2O_3 in other types of cancer (36). APL is characterised by a chromosome translocation generating a promyelocytic leukaemia-retinoic acid receptor α (PML-RARα) fusion gene whose product is degraded by As_2O_3 (29). Moreover, As_2O_3 (4 mg/kg bw; 30 min pre-treatment) was recently found to beneficially affect a murine model of asthma by inhibiting NFκB activity (172). Furthermore, As_2O_3 treatment (5 mg/kg bw; daily for 2 months) reduced symptoms and prolonged survival rates in a mouse model of the autoimmune disease systemic lupus erythematosus (19).

Arsenicals and infections

The effects of arsenicals on infections have been studied for several viruses. Almost 50 years ago, 0.1 mM of arsenite was found *in vitro* to reduce foot-and-mouth disease virus yield, but without causing permanent damage to the cells (133). More recently, As_2O_3 (<1 μM) was found to reduce viral replication in different *in vitro* models of hepatitis C infection, possibly through modulation of the glutathione redox system and oxidative stress (72, 101). Burkham et al. found that 30 min of pre-treatment with As_2O_3 (0.5-5μM) *in vitro* stabilised a nuclear domain normally disrupted during HSV-1 replication and suggested that As treatment could prevent the earliest stages of HSV-1 infection (24). At this concentration, cells were still dividing, although with slightly longer doubling times. Furthermore, a 2 h pre-treatment with arsenite (2.5-25μg/ml) *in vitro* dose-dependently decreased viral yield of bovine herpes virus-4 by inhibiting the expression of an early viral gene (IE-2) (87).

Contradictory to these results, several studies have reported an aggravated viral infection after concomitant exposure to arsenicals. As_2O_3 (2.5-15μM; treatment for the first 12 h of infection) was found to stimulate HIV-1 titres *in vitro* in some cell lines, but not in others (144). In addition, As_2O_3 (2μM) increased HIV-1 pro-virus after a single round of infection (18) and 16 h of pre-treatment with As_2O_3 (4μM), but not treatment 12 h post-infection, increased the levels of pro-viral HIV-1 DNA (158). Thus, it was concluded that As is likely to affect a viral replication step before, and not after, integration (18). Furthermore, low-dose As in the water (2 to 10 μg/l) of the zebra fish impaired the innate immunity, as shown by an increase in both viral and bacterial load and a decrease in several antiviral cytokines (127). Moreover, it was recently reported that 5-week exposure to As through drinking water (100 μg/l) resulted in higher pulmonary influenza A (H1N1) titres in mice (98).

Evaluation of the effects of arsenicals, both beneficial and toxic, is somewhat complicated because of the wide variation in cell types and *in vivo*

models, formulas of As and dosing regimens used. Thus, the effects of As on host defence and immune status need further investigation.

Enteroviruses

Enteroviruses (EVs), from the family picornaviridae, are small, non-enveloped positive-stranded RNA viruses with a genome size of approximately 7.5 kb (139). Human enteroviruses (HEVs) are categorised into HEV A to D, and the most important viruses in this family are the polioviruses, coxsackie A and B viruses and echoviruses (25). The polioviruses are included in HEV C, echoviruses and coxsackie B viruses are classified as HEV B, and the coxsackie A viruses are classified as either HEV A, B or C (25).

Antibodies to some of the viruses in group HEV B are found in up to 80% of adults, indicative of a very common human infection (139). However, as much as 50 to 80% of all non-polio EV infections are asymptomatic (123). Despite this, it is estimated that between 10 and 15 million people in the USA annually develop symptomatic non-polio EV infections (154). Although EV infections occur in all ages, infection rates are substantially higher in infants (139).

In temperate climates HEV infections appear with a marked summer and fall seasonality; the primary mode of transmission is the faecal-oral route. EVs are shed from the upper respiratory tract for 1-3 weeks and in the stool for 2-3 months after an infection (139, 140).

Infection and replication of human enteroviruses

EVs are believed to enter the host organism via the enterocytes in the intestine where they reach the Peyer's patches in the intestinal lumen. Here, significant viral replication occurs, leading to primary (minor) viremia where tissues (e.g., the CNS, liver, lungs, pancreas and heart) are infected. At these sites further replication occurs, leading to a second spread of a newly replicated virus and concomitant development of clinical signs and symptoms of disease (140).

EVs are believed to attach to cells through attachment to specific cell-surface receptors; several EV receptors have been identified, i.e. the poliovirus receptor (120), the intracellular adhesion molecule 1 (ICAM-1) (135), the decay-accelerating factor (DAF) (128, 147) and the human Coxsackie and adenovirus receptor (CAR) (17). After cell attachment, the viral structure is destabilised and uncoated RNA is released into the cytoplasm, where it rapidly binds to the ribosome for subsequent protein synthesis of a single polypeptide. The peptide is then cleaved by viral proteases into all the viral protein products, such as protease and structural proteins (140). At the same time, host protein synthesis is down-regulated, which facilitates the production of viral proteins (65). Infectious virions are then released through cell

lysis and/or by other non-lytic mechanisms and then spread to new cells for continued infection (94).

Coxsackievirus B (CVB) may control apoptosis of the infected cell by several possible mechanisms. These mechanisms include generation of double-stranded RNA, binding of cellular pro-apoptotic molecules and release of cytokines and chemokines as a result of NF-κB activation (71).

NFκB is found in almost all mammalian cell types and most of the inducible host defence genes are critically regulated, at least in part, by the NFκB pathway (118). NFκB controls the activation of a great number of cellular pathways resulting in expression of cytokines and enzymes and to a subsequent amplification of inflammatory responses, making NFκB a good target for new types of anti-inflammatory treatments (115).

One of the proinflammatory cytokines is IFN-γ that is produced mainly by natural killer cells and T-lymphocytes in response to viral infections and that has been found to affect viral infections at several levels – changed expression of viral receptors on cell surface, down regulation of viral regulatory elements and triggering of dsRNA kinase, i.e. obstruction of viral progeny production (40). The gene expression of IFN-γ was found to be reduced during Se deficiency and concomitant CVB3 infection in mice (10), further emphasising the importance of trace elements for the immune system.

Coxsackievirus B

Among the most common HEVs are the CVBs (140). The coxsackieviruses are named after the town where a member of this virus group was first isolated from a patient, i.e. in Coxsackie, New York, in 1948 (119). There are six CVB variants, CVB1 to CVB6 (25).

In mice infectious CVB3 has been isolated from the blood already within 24 h after inoculation, but not after day 3 (136, 167). In tissues infectious virus usually peaks on day 3 to 4 after infection and remains detectable up to 7 to 10 days after inoculation (136, 167). Viral RNA was shown to peak in the heart and pancreas about day 3 of a CVB5 infection in mice and was still detectable on day 10 of the infection (125). Furthermore, CVB3 RNA was detectable in tissues even 90 days after inoculation in mice (138). In the same study viral RNA was undetectable in the blood after day 14 (138). In other coxsackievirus infections in mice viral RNA was found to persist up to 12 months in muscle and pancreatic tissues (145, 156). From these findings, there is reason to believe that long-term persistence of viral RNA may occur in coxsackievirus infections under certain conditions.

Complications of enterovirus infections

Although most cases of non-polio EV infections are asymptomatic or mild, e.g., hand-foot-and-mouth disease, they are associated with several poten-

tially severe complications, including myocarditis, aseptic meningitis, encephalitis and pancreatitis (139, 140). EV infection has also been hypothesised to be one of several potential factors in inducing type 1 diabetes mellitus (140). Less serious, but for socioeconomic reasons very important, is the fact that EVs are responsible for approximately 15% of upper respiratory tract infections (30).

Although several viruses and bacteria can cause myocarditis, EVs, and in particular CVB, are the most commonly identified microorganisms (140, 165). One serious complication of myocarditis is dilated cardiomyopathy (DCM), which is believed to be the underlying cause of about 45% of all heart transplantations in the western world (70). Figure 2 shows CVB3 in muscle tissue.

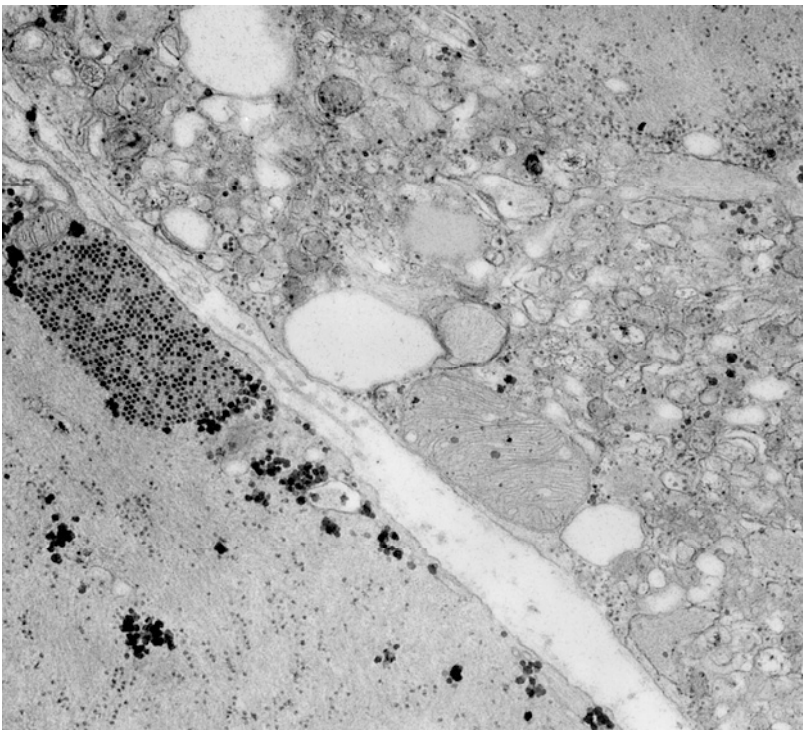

Figure 2. Transmission electron micrograph of coxsackievirus B3 particles within a specimen of muscle tissue. Photograph from the open database Public Health Image Library, Centre for Disease Control and Prevention (CDC), USA.

Other important complications following coxsackievirus infection is meningitis and encephalitis (140). EV meningitis in children above neonatal age and in adults is a common but relatively benign self-limited illness (140). EV encephalitis is less frequent, but in neonates EV infections during the first week of life can produce severe systemic infection, including meningoencephalitis, with a deadly outcome in 10% of the cases (140).

Although still controversial, EVs, and in particular CVB4, have been implicated as an initiating factor in type-1 diabetes (48, 49, 93). This research was stimulated by the fact that the pancreas is one of the target organs of EV infections and by a report of a child who died of diabetic ketoacidosis and from whose pancreas CVB virus was isolated and found to induce hyperglycaemia and beta-cell necrosis when inoculated into mice (169). EVs have been proposed to initiate or maintain autoimmune events in the pathogenetic processes of many cases of type-1 diabetes mellitus (45). However, because studies in humans are inconsistent they are not considered to provide final evidence for EVs as initiating factors in type-1 diabetes (63).

Anti-enteroviral therapy

It is evident that EVs can cause serious infections and complications, but today no antiviral drug that specifically targets these acute viral infections is available. *In vitro* studies of viral replication have shown beneficial effects of interferons, immunoglobulins and capsid-inhibiting compounds, as well as of protease inhibitors. However, attempts to validate these beneficial effects in clinical trials have failed, because of either poor efficacy or because of adverse side effects of the drug treatment (140).

The drug Pleconaril and its predecessors were found to have beneficial effects on EV replication (16, 47, 164). Because of adverse side effects, this drug, intended to be used in the treatment of the common cold, was not approved by the Food and Drug Administration (FDA) (164). In Europe and USA there are presently no therapeutic agents specifically registered for treatment of EV infections, although two drug candidates are currently being tested in phase II (34).

RNA viruses are known to rapidly mutate, and because at least one strain of EV was found to be resistant to the antiviral drug Pleconaril, a combination of different drugs would probably offer the best treatment strategy for serious EV infections (16, 164). It is therefore an urgent issue to find new, safe and effective therapeutic agents for these infections. Both essential and non-essential trace elements can change the pathogenesis of an infection (86, 88, 97, 101) and it is therefore believed that trace elements alone or in combination with other drugs potentially could be a future strategy to treat these infections.

Aims

The general aim of this thesis was to investigate the influence of trace elements, and in particular of As, on the replication of CVB3. The specific aims were the following:

- To study viral dissemination and associated changes in trace elements in serum and tissues during an acute CVB3 infection in mice (paper I)

- To investigate the effects of As_2O_3 treatment on serum and tissue trace element balance in healthy mice (paper II)

- To investigate whether As_2O_3 treatment affects serum and tissue trace element balance differently in healthy compared with CVB3-infected mice (paper III)

- To study the effects of As_2O_3 on virus replication in serum and tissues of CVB3-infected mice and *in vitro* (paper IV)

- To investigate the effects of As_2O_3 treatment on immune-mediated mechanisms in terms of gene expression of IFN-γ and NFκB in tissues of CVB3-infected mice (paper V)

Materials and methods

Virus

A myocarditic strain of CVB3 was previously propagated in HeLa cells and virus titres were determined as plaque-forming units (pfu) (46, 77). The same stock solution of virus has been used by our research group for a large number of studies on this infection, making it a well-characterised and standardised disease model. The stock solution of 10^7-10^8 pfu/ml, stored at -70°C, was diluted in sterile phosphate buffered saline (PBS) to $1x10^4$pfu/ml on the same day as the experiments (paper I, III, IV and V).

Arsenic test solution

A stock solution (10 mg/ml) of As_2O_3 was obtained from Ultra Scientific, USA. The stock solution was diluted in sterile saline to a final concentration of 0.125 mg/ml for the *in vivo* experiments (paper II, III, IV and V) or in cell culture medium to 0.4, 2 and 4 μM for the *in vitro* experiment (paper IV).

In APL As_2O_3 (Trisenox™) is approved by the FDA as induction treatment in doses of 0.15 mg/kg to be administered daily for a maximum of 60 days (43). Even though these patients are critically ill, As_2O_3 treatment is generally well tolerated, with adverse events generally self-limiting and reversible (142). Thus, the *in vivo* dose of As_2O_3 (1 mg/kg bw for 7 days) in the present study was in the same range as the dose for treatment of APL in humans.

Mouse model

The present experimental mouse model of CVB3 infection is well described and established for studies of interactions among infections and trace elements (81, 167). The murine infectious model of CVB3 shows a disease development similar to that in humans, where target organs (e.g., the pancreas, heart and brain) are infected and associated inflammatory lesions are induced by mobilised immune cells (42, 167).

The outcome of the murine CVB3 infection is dependent on both the viral strain and the mouse strain used. Both myocardial and amyocardial strains of

CVB3 exist (167). Besides nutritional status of the host, the sex and age of the host are important factors for the outcome of CVB3 infections (59, 167). Subsequently, male Balb/c mice infected with CVB3 have been found to be more susceptible to the development of pathologic lesions and mortality than female mice (59).

In the present studies of interactions between CVB3 and trace elements we used female Balb/c mice, aged 8-10 weeks (Charles River, Denmark), to study a relatively mild infection of CVB3 (46, 77, 167). Infected and control mice were maintained in separate cages at the animal department, Biomedical Centre, Uppsala, Sweden. Water and regular chow diet (Labfor R36; Lantmännen, Sweden) were supplied *ad libitum*.

The animal experiments took into account all ethical aspects of the welfare of animals following the recommendations in "Guide for the Care and Use of Laboratory Animals (CFN)". The studies were approved by the Ethical Committee for Animal Experiments, Uppsala, Sweden.

Cells

The effects of As_2O_3 on viral replication were studied *in vitro* (paper IV). Vero cells were incubated in Eagle's minimum essential medium supplemented with L-glutamine, antibiotics (Penicillin and Streptomycin) and 2% Foetal Bovine Serum at 36°C in 5% CO_2 in order to reach the desired confluency of 80-90% (i.e. approximately $5x10^4$ cells/ml were doubled in 20-24 h).

Experimental design

All *in vivo* studies were based on serum and tissue samples from different groups of mice from the same experimental study. In paper IV and V the same samples were used for different analyses.

Infection and treatment

On day 0 of the study, female Balb/c mice were either inoculated intraperitoneally with approximately $2x10^3$ pfu of CVB3 in 0.2 ml physiological saline or sham-inoculated with the same volume of saline. The mice were then treated once daily with intraperitoneal injections of 1 mg As_2O_3/kg bw in 0.2 ml physiological saline or with only saline. Altogether, the data set consisted of four groups of mice: non-infected untreated control mice, non-infected As_2O_3-treated mice, CVB3-infected untreated mice and CVB3-infected As_2O_3-treated mice.

On days 3, 5 and 7 of the study, six mice from each study group were sacrificed. The control group consisted of two mice from each time point, i.e. a total of six mice. Sham-inoculated and saline-treated mice were concomitantly sacrificed to serve as controls.

Paper I

Samples from CVB3-inoculated and sham-inoculated control mice were used. The trace elements Fe, Cu, Zn, As and Se were measured by inductively coupled plasma mass spectrometry (ICP-MS) in serum, heart, lung, liver, pancreas, kidney, spleen, intestine and brain. Changes in trace elements were related to the amount of CVB3 RNA, measured with quantitative reverse transcriptase PCR (RT-PCR) in the same tissues.

Paper II

Samples from non-infected As_2O_3-treated and untreated control mice were used. The effects of As_2O_3 treatment on magnesium (Mg) and the trace elements Fe, Cu, Zn, As, Se, Cd and Hg were studied (by ICP-MS) in serum, heart, lung, liver, pancreas, kidney, intestine and brain.

Paper III

The effects of As_2O_3 treatment on the trace element balance were studied in CVB3-infected and non-infected mice. Mg and the trace elements Fe, Cu, Zn, Se and As were measured (by ICP-MS) in serum, heart, lung, liver, pancreas, kidney, intestine and brain. To verify infection and the course of the disease CVB3 RNA was measured by RT-PCR in serum.

Paper IV

In vivo

The effects of As_2O_3 on viral replication were studied in CVB3-infected mice, i.e. in samples from As_2O_3-treated and untreated infected mice. CVB3 RNA was measured (by RT-PCR) in serum, heart, lung, liver, pancreas, kidney, spleen, intestine and brain. The concentration of the administered As in these organs was measured by ICP-MS.

In vitro

In paper IV an *in vitro* experiment was included in the experimental setup. Trypan blue staining and cell counting had indicated that As_2O_3 up to 3.2 μM was well tolerated during the study period of 5 days, whereas 4 μM resulted in a slightly slower growth rate. Thus, in the present study CVB3-infected cells were treated with 0.4, 2 or 4 μM of As_2O_3 (i.e. concentrations just below, within and slightly above the therapeutic range for As_2O_3 in APL cells) (29).

A CVB3 suspension containing $5x10^4$ pfu/ml was prepared in cell culture media containing 0.4, 2 or 4 μM of As_2O_3 and in media without As_2O_3 (for the untreated, infected control cells). Cells (approx $1x10^5$/well) were infected at a multiplicity of infection (MOI) of 0.1 (with or without As_2O_3) at 36°C in 5% CO_2 for 60 min. Unbound virus particles were washed away with PBS and thereafter fresh medium (with or without As_2O_3) was added to the cells. The cells were then incubated at 36°C in 5% CO_2 and checked once daily for cytopathogenic effects (CPEs). Mock-infected cells were used as non-infected controls.

On days 3 and 5 after infection, cell culture medium was carefully aspirated and cells were scraped. These two fractions were stored in separate sterile tubes at -70°C until quantification of CVB3 RNA as measured by RT-PCR.

Paper V

The aim of the study was to elucidate whether the antiviral effects of As_2O_3 were associated with immune-mediated mechanisms. The same study groups as in paper IV were used (i.e. CVB3-infected As_2O_3-treated and infected untreated mice) for studies of the gene expression of NFκB and IFN-γ, in the liver, pancreas and brain as measured by real-time PCR.

Tissue sampling

Mice were euthanised with Fluothane (Baxter Medical, Sweden). The thoracic cavity was opened and blood was collected from the heart using a heparinised syringe. Thereafter, the tissues from the thoracic and abdominal cavities were excised, and finally the skull was opened and the brain excised.

The tissue samples for PCR analysis were soaked in RNAlater (Qiagen, Sweden) and then stored at -70°C until RNA extraction was performed. The remaining part of the tissues was stored in tubes at -20°C until determination of trace element concentrations. Serum was separated from whole blood by centrifugation and then frozen together with the other tissue samples.

Molecular biology

RNA extraction

Isolation of total RNA from the tissues (approximately 25 mg) was performed using the TissueLyser (Qiagen) and RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's instructions (paper I, III, IV and V). Total nucleic acid from serum, culture medium and cells was isolated using the NucliSens® easyMAG (Biomeriéux, the Netherlands) (paper I, III and IV).

Detection of coxsackievirus B3

Real-time RT-PCR conditions, targeting a conserved region of the 5'UTR of the EV genome, have been described (124) and modified (39) elsewhere. The reaction was further adjusted by using 2.5U of r*Tth* polymerase (instead of 1.5U) and by running the reaction in a total volume of 25 µl (instead of 50 µl).

A standard curve obtained using a dilution series of a CVB3 cDNA-containing plasmid was used to estimate the number of CVB3 genome equivalents in each sample.

Samples from sham-inoculated mice were run in order to exclude contamination during infection/housing of the mice (paper I, III, IV and V).

Gene expression of NFκB and IFN-γ

All RNA samples were DNase treated (DNAfree, Applied Biosystems) and cDNA synthesis was performed using the Omniscript Reverse Transcription Kit (Qiagen) with Oligo-dT primers according to the manufacturers' protocols.

Gene expressions of NFκB and IFN-γ were analysed with real-time PCR (paper V) as previously described (111). Primers and probe sequences are shown in Table 1. Random samples of RNA (i.e. not reverse transcribed cDNA) were included in the PCR runs to control for genomic DNA. A PCR targeting glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was run on all cDNA samples to confirm intact cDNA.

Total RNA was used to normalise the gene expressions in all samples before a relative comparison of group means was performed.

Table 1. *Primer and probe sequences used for the IFN-γ and NFκB PCR.*

IFN-γ	
Forward primer	5'-CCTGCGGCCTAGCTCTGA-3'
Reverse primer	5'-AAGAGCTGCAAAGCCAAGATG-3'
Probe	5'-ACAATGAACGCTACACACT-3'
NFκB	
Forward primer	5'-CGGATGACAGAGGCGTGTATT-3'
Reverse primer	5'GTAGATAGGCAAGGTCAGAATGCA-3'
Probe	5'-CTATAATCCTGGACTTCTG-3'

Assessment of trace elements

The trace element concentrations in serum and tissues were determined by ICP-MS (Perkin-Elmer SCIEX ELAN 6000, Perkin Elmer Corp., Canada) as previously described (51).

For quality control, every eighth sample was a certified reference material: Human whole blood (batch MR4206) and serum (batch JL4409), both from Sero AS, Norway; bovine liver (1577a; National Institute of Standards and Technology, USA), bovine muscle and pig kidney (Community bureau of reference, Belgium) and IAEA H-4 animal muscle (International Atomic Energy Agency, Analytical Quality Control Services, Austria).

Statistical analysis

A one-way analysis of variance (ANOVA) was performed to determine whether there were any differences between the study groups. When the null hypothesis was rejected, Dunnett's method (paper I and II) or Tukey's method (paper V) for multiple comparisons was adopted.

When the samples were not normally distributed, or when the assumption of homogeneity in group variances were not fulfilled, the ANOVA was not applicable. In these cases the Kruskal-Wallis (a non-parametric ANOVA) was performed (paper I, II, III and V) and followed by multiple comparisons. In paper III and IV Mann-Whitney's U test was used to compare two independent groups.

To investigate a possible correlation between the numbers of CVB3 equivalents and trace element concentrations Spearman rank correlations were calculated (paper I).

Results

CVB3 infection and trace element balance (paper I)

Several studies have reported significant changes in trace elements during viral infections though these changes have never been correlated to viral load. Furthermore, the present study aimed to describe the infection-induced changes in trace elements in not only target organs of the infection but also in serum and other infected tissues.

Experimental mouse model

Infected mice developed expected clinical symptoms (e.g., ruffled hair and inactivity) from day 2 of the infection.

Quantification of viral RNA

Viral RNA was detected in all tissues in all infected mice (Table 2). In most of the studied tissues the concentration of viral RNA peaked on day 3 of the infection, although a delayed response was seen in the heart, intestine and brain. In the heart and intestine, viral RNA load tended to peak on day 5 of the infection, whereas viral RNA in the brain tended to increase sequentially during the 7 days of the study. On day 7 of the infection, the amount of viral RNA was significantly lower than on day 3 in all organs except the heart, intestine and brain. Moreover, the viral RNA load in the pancreas was approximately 20 times higher than in the other organs.

All sham-inoculated control mice were found negative for CVB3 RNA.

Table 2. *Number of CVB3 copies in serum and tissues on days 3, 5 and 7 of infection.*

CVB3 copies per mg tissue x 1000 in serum and tissues			
Organ	**Day 3**	**Day 5**	**Day 7**
Serum[a]	1 200 (2 020)	12 (17) **	0.7 (1.3) ***
Heart	7 020 (2 860)	13 520 (7 740)	4 250 (3 630)
Lung	837 (391)	156 (175) **	22 (23) ***
Liver	4 150 (2 550)	1 160 (1 210)	64 (116) **
Pancreas	241 000 (144 000)	198 000 (50 000)	96 000 (62 000) *
Kidney	7 660 (7 020)	823 (1020) *	417 (364) **
Spleen	11 600 (4 900)	1 160 (1 060) ***	826 (535) ***
Intestine	10 700 (11 600)	21 100 (26 600)	5 400 (11 500)
Brain	67 (92)	300 (700)	380 (190)

[a]Serum is quantified as virus equivalents per µl serum.
n=6 in each group (except for day 3, where n=5).
Data are expressed as mean and standard deviation (in parentheses). Asterisks on days 5 and 7 denote a significant difference (*$p<0.05$, ** $p<0.01$, *** $p<0.001$) in comparison with the viral RNA count on day 3.

Assessment of trace elements

The CVB3 infection resulted in a changed balance of trace elements in all studied tissues. The most consistent changes were a decrease in Se in all tissues except the heart, and a decrease in As in all tissues except the kidney, spleen and brain. Briefly, when compared with non-infected controls, the changes in Se and As in the target organs of the CVB3-infection, i.e. the heart, pancreas and brain, were as follows: In the heart As decreased by 63% (day 3; $p<0.05$). In the pancreas Se decreased by 15% (day 5; $p<0.05$), whereas As after an initial increase of 73% (day 3; $p<0.01$) decreased by 47% (day 5; $p<0.05$). Finally, in the brain Se decreased by 18% (day 7; $p<0.05$).

Moreover, a negative correlation ($p<0.05$) between viral RNA load and Se concentration was found in serum on days 5 ($r= -0.94$) and 7 ($r= -0.89$), in the liver on days 5 ($r= -0.89$) and 7 ($r= -0.89$), in the pancreas on day 7 ($r= -0.94$) and in the intestine on day 7 ($r= - 0.89$) of the infection.

As_2O_3 and trace element balance in healthy mice (paper II)

Study I indicated that not only Se, but also As was involved in immune processes and/or viral replication during the CVB3 infection. However, As has been shown to interact with the metabolism of Se (55, 104) and to induce metal-binding/transporting proteins (100, 112). Therefore, it was important to investigate the effects of As administration on the general trace element

homeostasis in non-infected mice before studies could be performed on a possible antiviral effect of As.

Experimental mouse model

No treatment-related adverse reactions were observed in the mice administered As_2O_3.

Assessment of trace elements

As_2O_3 treatment in healthy mice resulted in a changed balance of trace elements in some, but not in all, of the tissues studied. In fact, in the heart, lung and liver all studied trace elements remained unchanged, except the supplemented As. The concentration of As in the As_2O_3-treated mice was significantly higher ($p<0.001$) in all tissues at all three time points.

The most pronounced changes in essential trace element concentrations in As_2O_3-treated mice were a decrease (64%, $p<0.001$) in serum Zn on day 7 and a decrease in Se in the pancreas (9%, $p<0.05$) and the intestine (30%, $p<0.001$) on day 3. A decrease of Se was also noted in the brain on days 5 (12%, $p<0.05$) and 7 (15%, $p<0.01$).

As_2O_3 and trace element balance in infected mice (paper III)

In non-infected mice As caused only minor effects on the normal trace element balance in serum and studied tissues (paper II). However, an ongoing infection can change the distribution of an administered trace element (75, 79). Thus, it was important to investigate the effects of As administration on the general trace element balance in CVB3-infected mice before studies could be performed on a possible antiviral effect of As.

Experimental mouse model

No treatment-related adverse reactions were seen in the mice administered As_2O_3, regardless of concomitant CVB3 infection.

Assessment of trace elements

As_2O_3 treatment during CVB3 infection changed the concentrations of trace elements in all studied tissues differently as compared with changes in As_2O_3-treated healthy mice, with the most pronounced differences observed in Zn, As and Se. In the infected and As_2O_3-treated mice Zn increased in all tissues except the pancreas, where it decreased dramatically at all three time

points (i.e. days 3, 5 and 7) of the study (Table 3). Furthermore, in the infected mice As increased in all organs except the intestine, where it decreased when compared with non-infected mice (Table 4). In contrast, Se decreased in all organs except the heart and brain in the infected mice (Table 5).

Table 3. *Concentrations of Zn in serum and tissues of As_2O_3-treated, non-infected (As) and As_2O_3-treated CVB3-infected (As + CVB3) mice on days 3, 5 and 7.*

Zn concentration (µg/kg wet weight) in serum and tissues						
	Day 3		**Day 5**		**Day 7**	
	As	As + CVB3	As	As + CVB3	As	As + CVB3
Serum	667	1480	669	802	299	399
	(169)	(274)**	(111)	(222)	(98)	(145)
Heart	19 400	21 400	20 400	33 200	22 300	24 500
	(1000)	(900)**	(1 500)	(3 500)**	(2 800)	(3 600)
Lung	15 800	19 100	15 700	17 100	15 500	14 600
	(1 600)	(800)**	(2 000)	(400)	(1 700)	(1 000)
Liver	30 800	38 400	30 900	35 900	30 400	45 600
	(700)	(6 400)	(1 500)	(7 400)	(1 800)	(15 000)**
Pan-	37 500	13 800	34 000	15 100	38 400	20 100
creas	(5 100)	(300)**	(3 300)	(4 300)**	(5 800)	(7 300)**
Kidney	17 600	18 100	18 500	47 500	16 100	18 500
	(800)	(1 600)	(800)	(20 700)*	(1 700)	(700)*
Intes-	14 500	13 400	12 600	12 500	12 300	13 900
tine	(1 800)	(1 900)	(600)	(1 500)	(1 100)	(1 100)*
Brain	18 600	18 800	18 600	18 600	17 200	18 400
	(2 500)	(1 100)	(1 400)	(300)	(400)	(600)**

n = 6 in each group.
Data expressed in mean (standard deviation).
Asterisks denote a significant difference (*p<0.05, **p<0.01) between non-infected and CVB3-infected mice on days 3, 5 and 7, respectively.

Table 4. *Concentrations of As in serum and tissues of As_2O_3-treated, non-infected (As) and As_2O_3-treated CVB3-infected (As + CVB3) mice on days 3, 5 and 7.*

As concentration (µg/kg wet weight) in serum and tissues						
	Day 3		**Day 5**		**Day 7**	
	As	As + CVB3	As	As + CVB3	As	As + CVB3
Serum	8	14	6	13	10	13
	(1)	(5)**	(2)	(3)**	(5)	(2)
Heart	89	105	124	165	133	199
	(7)	(17)*	(19)	(47)	(22)	(17)**
Lung	93	126	102	160	124	185
	(25)	(30)*	(12)	(33)**	(20)	(20)**
Liver	240	248	296	316	302	414
	(20)	(27)	(41)	(53)	(41)	(38)**
Pancreas	63	410	82	302	84	135
	(12)	(161)**	(15)	(84)**	(16)	(19)**
Kidney	130	131	160	168	176	223
	(12)	(24)	(33)	(51)	(35)	(24)*
Intestine	82	64	101	69	105	99
	(16)	(12)	(15)	(23)*	(27)	(8)
Brain	14	19	18	31	21	36
	(2)	(5)	(3)	(16)	(3)	(3)**

n = 6 in each group.
Data expressed in mean (standard deviation).
Asterisks denote a significant difference (*$p<0.05$, **$p<0.01$) between non-infected and CVB3-infected mice on days 3, 5 and 7, respectively.

Table 5. *Concentrations of Se in serum and tissues of As_2O_3-treated, non-infected (As) and As_2O_3-treated CVB3-infected (As + CVB3) mice on days 3, 5 and 7.*

Se concentration (µg/kg wet weight) in serum and tissues						
	Day 3		**Day 5**		**Day 7**	
	As	As + CVB3	As	As + CVB3	As	As + CVB3
Serum	247	213	222	187	267	247
	(19)	(15)**	(16)	(19)**	(29)	(29)
Heart	276	246	257	242	232	243
	(51)	(11)	(21)	(18)	(21)	(17)
Lung	1 200	1 000	1 200	1 100	1 200	1 000
	(100)	(100)*	(100)	(200)	(100)	(100)**
Liver	1 400	1 100	1 400	1 000	1 300	1 100
	(100)	(100)**	(100)	(200)**	(100)	(100)**
Pancreas	421	400	449	409	469	495
	(34)	(21)	(19)	(18)*	(20)	(47)
Kidney	1 700	1 600	1 800	1 600	1 900	1 700
	(100)	(100)	(100)	(100)**	(100)	(100)**
Intestine	166	142	237	150	220	223
	(26)	(13)	(14)	(17)**	(21)	(25)
Brain	224	237	218	240	211	228
	(12)	(32)	(10)	(30)	(8)	(21)

n = 6 in each group.
Data expressed in mean (standard deviation).
Asterisks denote a significant difference (*p<0.05, **p<0.01) between non-infected and CVB3-infected mice on days 3, 5 and 7, respectively.

In the CVB3-infected mice the daily administration of As_2O_3 resulted in a sequential increase in As in all tissues except the pancreas. Surprisingly, the As concentration in this tissue tended to decrease over time despite daily treatment of As_2O_3. In the pancreas of the non-infected mice the lowest As concentration was found on day 3 followed by a tendency of a gradual increase until day 7. This same pattern was also observed in the other tissues in the non-infected mice. Another noteworthy finding was that, in comparison with the non-infected mice, Mg in the serum of the infected mice transiently increased on days 3 (8%; $p<0.05$) and 5 (41%; $p<0.01$), whereas Mg in the heart decreased (11%; $p<0.01$) on day 7.

As_2O_3 and antiviral effects (paper IV)

The previously shown infection-induced decrease in As in most tissues, together with the finding of an increased tissue uptake of supplemented As during a concomitant viral infection, indicates that As could be involved in

viral replication. Antiviral effects of As_2O_3 in the present study were investigated in both *in vitro* and *in vivo* experimental models of CVB3 infection. In both model systems the antiviral effect was measured as a decrease in viral RNA.

Experimental mouse model

On day 3 of the CVB3 infection, both As_2O_3-treated and untreated mice had developed clinical signs of disease (e.g., ruffled hair and inactivity). Although these effects were less pronounced in the As_2O_3-treated mice, As_2O_3 treatment did not affect early CVB3-induced mortality.

Quantification of viral RNA in serum and tissues

Viral RNA was found in all infected tissues on days 3, 5 and 7 of the infection. As_2O_3 treatment resulted in a decreased viral RNA load in the pancreas on day 7 (by 75%; $p<0.05$) and in the brain on days 3 (by 81%; $p<0.05$) and 7 (by 97%; $p<0.01$) of the infection. Thus, a decrease was seen in two of the target organs of the CVB3 infection (Figure 3). Furthermore, viral RNA tended to decrease in the heart on day 5 of the study, although this decrease was not statistically significant.

Viral RNA tended to increase throughout the 7 days of the study in the untreated infected brain, i.e. viral RNA tended to peak on day 7 of the infection (also reported in paper I). This finding contrasts to the pattern in the As_2O_3-treated infected brain, where viral RNA tended to peak on day 5 of the infection.

No viral RNA was found in any of the non-infected mice.

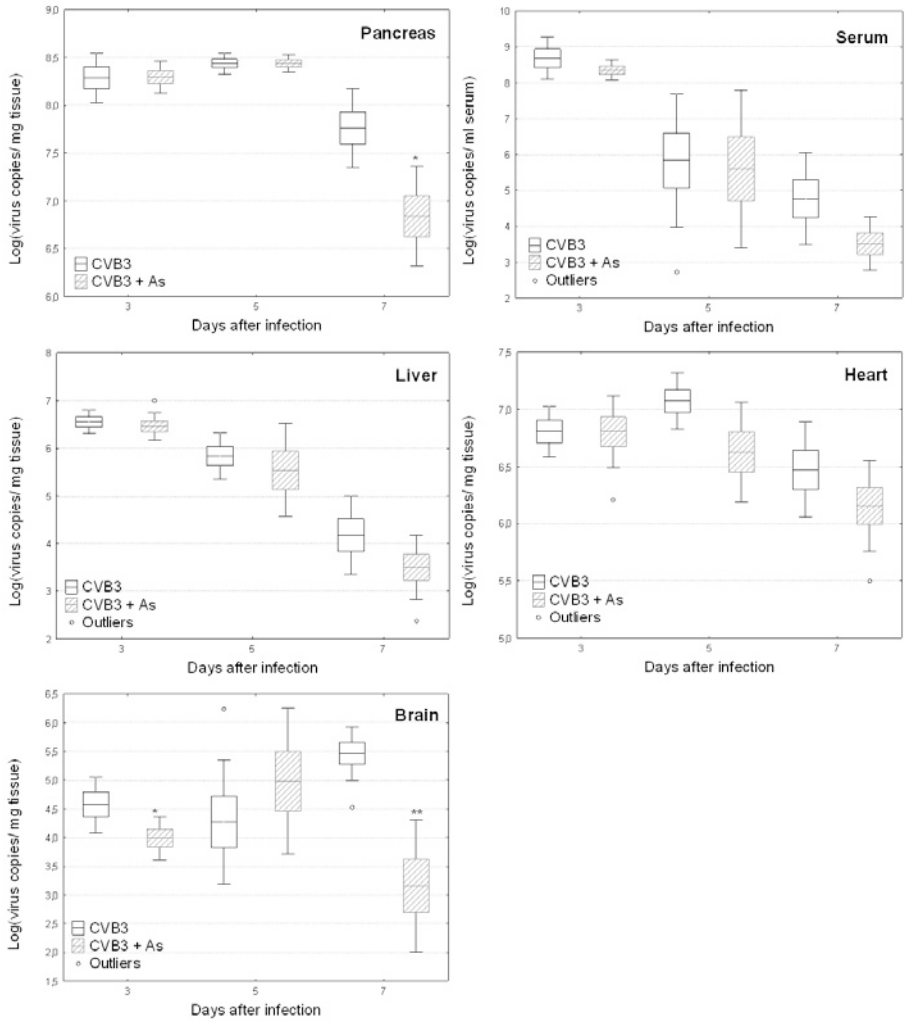

Figure 3. Amount of CVB3 RNA in serum and tissues of untreated and As_2O_3-treated mice on days 3, 5 and 7 of the study. The central thick line indicates mean virus RNA copies per mg tissue or ml serum, the box indicates mean ± standard error and the whiskers extend out to the range of the standard deviation. Asterisks denote a significant difference (* $p<0.05$, ** $p<0.01$) between untreated and As_2O_3-treated mice.

Assessment of As concentration in serum and tissues

When compared with untreated CVB3-infected mice, As_2O_3 treatment of CVB3-infected mice resulted in an increased As concentration ($p<0.01$) in all studied tissues on all three time points. Particularly notable was that, in

comparison with the other tissues, the increase in brain As after As_2O_3 treatment was relatively small.

Quantification of viral RNA in cells and culture medium

On day 2 of the *in vitro* study, the infected cells started to show the first signs of CPE. On day 5, massive CPE was found in all infected cells in both As_2O_3-treated and untreated groups.

As_2O_3 dose-dependently reduced CVB3 RNA concentrations on days 3 and 5 of the infection (Figure 4), although the effects of As_2O_3 treatment on day 5 were less pronounced. However, on day 5 of the infection, CVB3 RNA decreased in the culture medium at all three doses of As_2O_3 treatment, whereas CVB3 RNA in the cell fraction was increased at the two lower doses of As_2O_3, i.e. at 0.4 and 2 µM.

All control samples were found negative for viral RNA.

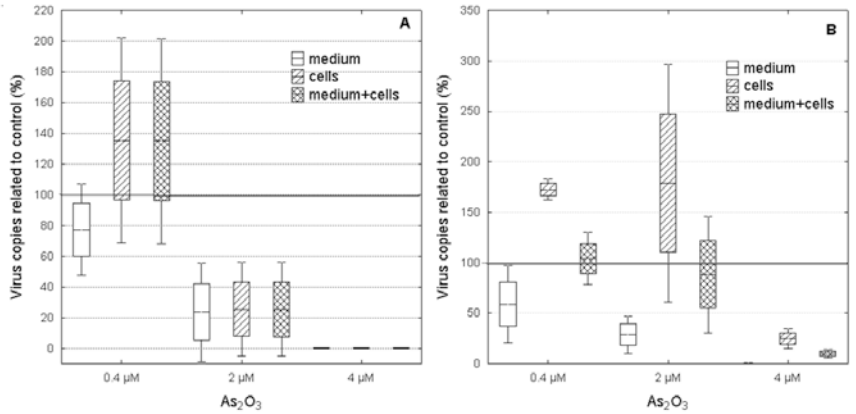

Figure 4. Changes in CVB3 RNA in cell culture medium and in cells on days 3 (A) and 5 (B) of the infection. All values are expressed as percentage change in As_2O_3-treated CVB3-infected compared with untreated CVB3-infected cells (100%).

As_2O_3 and immune mediators (paper V)

Based on our previous data on viral RNA load and As concentration in infected tissues, it was concluded that As influences viral replication. However, it could not be excluded that these effects were at least partly immune-mediated. Thus, in paper V immune-mediated mechanisms in terms of gene expression of IFN-γ and NFκB were studied in the pancreas and brain (i.e. the two organs where As_2O_3 significantly reduced viral RNA load) and in the liver (representing a metabolically active organ), where in the previous study no significant antiviral effect of As_2O_3 could be detected.

Gene expression of IFN-γ

As_2O_3 treatment resulted in an earlier ($p<0.05$) infection-induced response in the gene expression of IFN-γ in the brain. Four of six As_2O_3-treated mice were positive for IFN-γ in the brain already on day 5 of the infection (Figure 5). Untreated, CVB3-infected mice showed no gene expression of IFN-γ in the brain until day 7, where five of six mice were positive. As_2O_3 treatment did not affect the expression of IFN-γ in the liver or the pancreas, in the CVB3-infected and non-infected mice.

No expression of IFN-γ could be detected in the pancreas and brain of the uninfected groups, regardless of As_2O_3 treatment. This resulted in a significantly increased expression of IFN-γ in all three tissues (i.e. the liver, pancreas and brain) in the infected mice. Consequently, the infection had more impact on the expression of IFN-γ than As_2O_3 treatment.

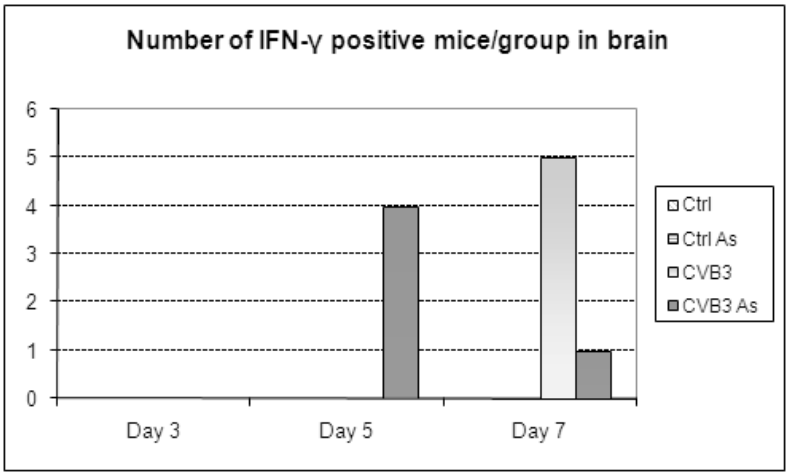

Figure 5. Number of mice positive for IFN-γ in the brain on days 3, 5 and 7 of the infection. Ctrl = non-infected untreated mice. Ctrl As = non-infected As_2O_3-treated mice. CVB3 = coxsackievirus B3-infected, untreated mice. CVB3 + As = coxsackievirus B3-infected As_2O_3-treated mice. n = 6 in each group (except for CVB3 on day 3 where n = 5).

Gene expression of NFκB

As_2O_3 treatment did not affect the infection-induced gene expression of NFκB in any of the studied tissues, i.e. the liver, pancreas and brain. On the contrary, in the uninfected brain As_2O_3 treatment resulted in a two cycle difference in quantification cycle (Cq) value, roughly corresponding to a six-fold increase in gene expression of NFκB ($p<0.01$; day 7), when compared with uninfected, untreated control mice (Figure 6). Thus, As_2O_3 treatment

affected the expression of NFκB in the brain of non-infected mice, but not CVB3-infected mice.

Similarly to the response in IFN-γ, the infection-induced expression of NFκB was not affected by As_2O_3 treatment in the liver or the pancreas. However, in all studied tissues the infection *per se* resulted in an increased expression of NFκB on at least one of the time points of the study ($p<0.05$). It therefore seems that CVB3 infection affects NFκB in target tissues on various time points of the disease.

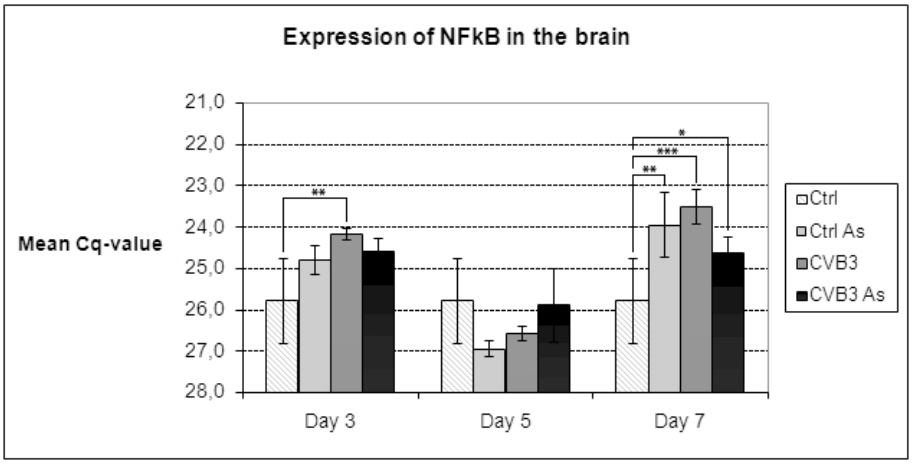

Figure 6. Gene expression of NFκB in the brain of As_2O_3-treated and untreated mice with or without concomitant CVB3 infection on days 3, 5 and 7 of the study. Abbreviations in the figure correspond to Ctrl = non-infected untreated. Ctrl As = non-infected As_2O_3-treated. CVB3 = coxsackievirus B3-infected, untreated. CVB3 + As = coxsackievirus B3-infected As_2O_3-treated. n = 6 in each group (except for CVB3 on day 3 where n = 5). Data expressed as mean PCR quantification cycle (Cq) and standard deviation. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Discussion

Several trace elements are essential for different arms of our immune defence and our host resistance to infections. A consequence of this is that the normal balance of essential trace elements is changed in serum and target organs of infections (80). Furthermore, both deficiency and excessive amounts of trace elements can adversely influence host resistance to a variety of pathogens (6, 11, 80, 149). However, different trace elements affect not only the invaded host but also the invading microorganism. For example, a lack of Zn that is caused by chelation can trigger cell death in virus-transformed cells (44). Moreover, Fe is an essential nutrient for many pathogens and the ability of the host to sequester Fe is believed to be a primary defence mechanism against several bacterial infections (37, 132). Metal ions are also important components in several gene regulatory proteins, including virus proteins (44) and seem to play an important role in the replication of human rhinoviruses (99). *In vitro* data suggest that As affects replication of picornavirus (133), but in CVB3-infected mice As was decreased in the target organs of active viral replication (15). In line with this observation is the finding that As increases the stability of the replication protein compartment that needs to be disrupted for successful replication and production of progeny HSV-1 (24) and inhibits hepatitis C virus replication, possibly by interfering with cellular factors necessary for replication (101). Thus, As seems to influence replication of certain viruses and possibly also the host defence or the replication of CVB3. However, because trace elements interact with each other, it is important to study how As influences the general balance of both essential and non-essential trace elements (in both health and infection) before a possible antiviral potential can be elucidated.

Viral dissemination during experimental CVB3 infection

The murine infectious model of CVB3 used in the present study shows a disease development similar to that in humans, i.e. involvement occurs in the target organs: the pancreas, liver, heart, meninges and brain (167). For this experimental infection, viremia is known to occur around day 2 to 3, whereas infiltration of inflammatory cells into the myocardium and the first

signs of myocarditis are apparent from day 6 to 7 of the infection (42, 167). Thus, the three time points for tissue sampling in the present study were on days 3, 5 and 7 of the infection, which was required to cover both disease stages of the CVB3 infection. To ensure that all mice received the same dose and to be able to make comparisons with findings of other investigators, intraperitoneal injections for the viral inoculations were used in the present study. This method is commonly used for experimental CVB infection (12, 42, 90, 125, 138), although it has been shown that the administration route may have some impact on the outcome of the infection (20).

The presently used CVB3 infection resulted in a transient viremia with a peak in viral RNA on day 3 of the infection in all studied tissues, except the heart, intestine and brain (paper I). Viral RNA in these tissues was not significantly reduced even after 7 days of infection when compared with viral RNA on day 3 of the infection. However, clinical symptoms were apparent already on day 2 of the infection.

Viral RNA load in the pancreas was markedly higher than in the other tissues. However, Kim et al. reported no correlation between CVB3 RNA copy number in pancreatic tissue and severity of inflammation (90).

Changes in trace elements during CVB3 infection

During the 7 days of the acute CVB3 infection, the balance of essential and non-essential trace elements was changed. The most consistent changes were found in Se, which decreased in all organs except the heart, and in As, which decreased in all organs except the kidney, spleen and brain. The decrease in As was in the same range as in previous studies of this infection (15, 52). Possibly, these changes are specific for viral infections because *C. pneumoniae* infection in mice did not significantly affect Se and As in serum or heart (38). Furthermore, in the present study a negative correlation was found between viral RNA and Se concentration in serum, liver, pancreas and intestine.

Other investigators have shown that an adequate Se status is important for a positive outcome of various infections of both viral and bacterial origin (9, 10, 21, 88). For example, Se deficiency in infected mice has been shown to increase the virulence of the infecting CVB3 through changes in the viral genome (12), whereas increased activity of a Se-containing protein recently was found to reduce the replication of HIV-1 *in vitro* (88). Se is a constituent of different selenoproteins and required for the functioning of neutrophils, macrophages, NK cells and T-lymphocytes (26). Moreover, Se is an essential part of the antioxidant enzyme glutathione peroxidase (26). It is thus evident that Se has a role in the host defence. Furthermore, Se supplementation has been found to reduce the development of inflammatory lesions that occur in CVB3-induced myocarditis (86). With this in mind, it appears that

Se may exert its antiviral effects directly by inhibiting viral replication and affecting cells of the immune system. However, several viruses (e.g., CVB3, hepatitis B and C, HIV-1 and 2) are capable of making viral selenoproteins and thereby reducing the host selenium supply (170, 171), indicating that supplementation of Se may not be an efficient anti-viral treatment strategy for all types of viral infection. Antiviral effects have also been reported for As compounds (24, 72, 133), although the results are somewhat contradictory (18, 57, 127, 158). As is therefore an intriguing alternative in the search for new antiviral therapies.

Effects of As_2O_3 on trace element balance

Several studies have confirmed the presence of As-Se bonds (54, 104). As given to Se exposed rats resulted in redistribution and enhanced excretion of Se (104). It is therefore very important to concomitantly study changes in Se during As therapy. Furthermore, As can specifically induce metal-binding/transporting proteins that could influence the binding and balance of other essential trace elements (22, 100, 112). As supplementation could therefore compete and interfere with trace elements essential for both host defence and replication of microorganisms. Thus, prior to studies of possible therapeutic effects of As on the replication of CVB3, it was important to investigate the effects of As_2O_3 on the general trace element balance in serum and tissues, both in non-infected and infected mice.

In non-infected mice administration of As_2O_3 resulted in a sequential reduction in serum Zn and a reduction in Se in the pancreas, intestine and brain. However, in the remaining tissues changes in trace elements were surprisingly minor and inconclusive. The change in serum Zn was likely due to induction of metallothioneins that are inducible by As and known to bind Zn (22, 100, 112), whereas a possible relationship between Se and metallothioneins is less clear and these changes in Se concentration likely have to be explained by another mechanism. However, As has been shown to stimulate the gastrointestinal excretion of Se when given within 1 h after the administration of Se (104). To the contrary, Se administration has been shown to increase the gastrointestinal excretion of As (104). This effect has been suggested to be caused by the formation of a detoxification conjugate between As and Se that could be excreted in the bile (56, 105). Such a mechanism may also explain the reduction in Se seen in the present study in the pancreas and intestine, as well as to some degree in the brain. However, it was rather surprising that 7 days of As supplementation in healthy mice had only a relatively minor effect on the Se concentration in serum and the other tissues.

When As_2O_3 was administered during the CVB3 infection, trace element balance was affected more extensively in the infected mice than in the

healthy mice (paper III), with the most pronounced differences found in Zn and Se. In the infected As_2O_3-treated mice Zn increased in all tissues except the pancreas, whereas Se decreased in all tissues except the heart and brain. In addition, in infected mice As_2O_3 treatment resulted in an increased As concentration in all tissues except the intestine, where the As concentration decreased compared with healthy mice. During CVB3 infection, it is well-known that non-essential trace elements (Ni, Cd, Hg) can be distributed quantitatively differently in the body as compared with the distribution in a healthy individual (50, 76, 79). Moreover, exposure of Ni and Cd during an infection can change the activity of immune cells and thus affect the host immune response (75, 78). Thus, the risk for potential adverse health effects of As may increase during treatment of an infectious disease or when inflammation is a part of the disease pathogenesis. This finding may be of great concern when As_2O_3 is used for cancer treatment and should influence the doses when As therapy is used in a clinical setting.

During the acute-phase response to infections, it is widely known that the concentration of essential trace elements can change (14). Accordingly, As_2O_3-induced decrease in Se was comparable with the CVB3-induced decrease (i.e. infection without As_2O_3) seen in paper I. Based on this finding, it is reasonable to assume that the decrease in Se was an effect of the infection *per se*, rather than an effect of the As_2O_3 treatment. The increase in Zn and As in infected compared with healthy As_2O_3-treated mice was probably the result of a synergistic increase in the expression of metal-transporting/binding proteins that is caused by both the CVB3 infection and the administration of As.

Prolongation of the QT interval on the electrocardiogram and ventricular tachycardia are some of the more severe adverse events reported during As_2O_3 treatment in leukaemia patients (151). The same phenomena can be the result of an electrolyte imbalance, mainly in Mg and calcium, and it has therefore been recommended to closely monitor this balance during As_2O_3 treatment in leukaemia patients (43, 151). In the present study As_2O_3 treatment in the healthy mice did not affect Mg in serum or in the heart. In contrast, in the infected mice As_2O_3 treatment resulted in a transient increase in Mg in serum but a decrease in the same trace element in the heart on day 7 of the study. Thus, serum levels of trace elements do not seem to reflect the tissue content of the element and may conceal an actual difference in the organ of interest.

Effects of As_2O_3 on viral replication

In the present study 7 days of As_2O_3 treatment during CVB3 infection in mice resulted in a decrease in viral RNA in the brain and pancreas, two of the target organs of this infection. This observation was confirmed *in vitro,*

where 0.4 to 4 μM of As_2O_3 reduced the viral RNA load in the cell medium surrounding the cells. Although the As_2O_3-treated mice had somewhat milder clinical symptoms (i.e. ruffed fur and inactivity), the treatment did not reduce CVB3-induced mortality.

A tendency of an increase in brain viral RNA in the As_2O_3-treated mice on day 5 coincided with a marked increase in the As concentration between days 3 and 5 in the brain. Au et al. suggested that oral As_2O_3 cannot efficiently penetrate the blood-brain barrier (BBB) until it is damaged – in that case by meningeal leukaemia (4). CVB3 infection induces a number of cytokines and chemokines (110) and it has been indicated that inflammatory cytokines affect certain metal proteinases, which, in turn, result in weakening of the BBB (109). Other investigators have reported on virus-associated damage of the BBB that is caused by viruses, such as tick-borne encephalitis (TBE) (64) and measles virus (31). Thus, the BBB may be directly influenced by the virus or by associated immune processes, presumably responding with an increased permeability to both microorganisms and chemical substances, including trace elements. A damaged barrier would thus allow CVB3 and As_2O_3 entry (indicated by the tendency of the increase in viral RNA and As on day 5) followed by an antiviral effect on CVB3 in the brain and thus a reduction in viral RNA in the As_2O_3-treated group on day 7 of the study. This antiviral effect of As_2O_3 in the brain is an important finding because it is sometimes difficult to reach therapeutic drug concentrations in the brain because of the protective effects of the BBB (130). An oral formulation of As_2O_3 has been found to cross the BBB and to reduce the peak plasma concentration of As (4, 148). Oral administration is thus a feasible alternative to the i.p. administration used in the present study in the pursuit of new antiviral therapies.

As compounds have been reported to impair replication of HCV (72, 101), HSV-1 (24) and foot-and-mouth disease virus (133), although studies on other viral infections, mainly HIV-1, have reported a stimulatory effect of As on viral replication (98, 144). In mice, western encephalitis virus infection-induced mortality was reduced when As was administered at the time of virus inoculation, whereas As administration during ongoing disease increased the mortality, suggesting that the timing of administration is important for the effects of As during an infection (57). As_2O_3 treatment in the present study was initiated at the time of infection, both *in vivo* and *in vitro*. Furthermore, it has been shown that the effect of As_2O_3 on viral infectivity differs between different strains of cells (144). Thus, a cell- or tissue-specific effect of As may explain some of the differences seen in ours and other studies.

As_2O_3 treatment *in vitro* resulted in reduced viral RNA load in the cell culture medium, whereas viral RNA in the cell increased for the two lower drug concentrations when compared with untreated, infected cells. Although the highest of the As_2O_3 doses tested *in vitro* slightly reduced the growth rate

of the Vero cells (when stained with trypan blue), the observed increase in viral RNA in the cells would suggest that the antiviral effect is caused by impairment in virion packaging and/or release rather than impairment on the cellular replication machinery *per se*. Furthermore, others have shown in other cell systems that TD50 of As_2O_3 after 1 and 3 days of treatment was 6.3 and 12.3 μM, respectively (72), indicating adaptation to a higher dose. The antiviral effect of As_2O_3 on hepatitis C was suggested to be mediated by a decrease in the intracellular levels of glutathione, which would affect oxidative stress (101). Moreover, glutathione was found to be required for efficient production of infectious picornavirus virions (150). Taken together, these findings further support the hypothesis of an impaired release of infectious virus.

Effects of As_2O_3 on immune mediators

Infections with several different viruses have been found to activate NFκB, probably as a mechanism to delay apoptosis in order to ensure sufficient time for virus replication, and consequently, suppression of NFκB activation was found to block CVB3 RNA synthesis and progeny virion release in infected cells (41). In addition, Mathas et al. concluded that inhibition of NFκB contributed to As-induced apoptosis (114). The authors further concluded that NFκB in cell lines lacking an active IκB (i.e. an inhibitor of NFκB) was unaffected, resulting in cells resistant to As-induced apoptosis (114). Thus, the hypothesis of the present study is that the antiviral effect of As_2O_3 could be a consequence of an inhibition of NFκB. However, no effect of As_2O_3 treatment on NFκB was found in the CVB3-infected liver, pancreas and brain in the present study. A dual effect of As has been shown in which low levels of As seem to increase the transcription of NFκB, whereas high levels of As inhibit the activation of NFκB (27, 35). This observation implies that there is a delicate balance between possible beneficial and detrimental effects of As, i.e. the therapeutic window may be narrow. The results from the present study were in agreement with *in vitro* findings by others that the beneficial effect of As_2O_3 (1μM) on hepatic C virus replication and on APL was unrelated to NFκB activity (27, 101). However, As_2O_3 treatment did increase the gene expression of NFκB in non-infected mice, showing that supplementation may affect the trace element balance differently in health compared with disease.

IFN-γ, which is activated during viral infections, is an important component of the innate early immune response (40). In the present study As_2O_3 treatment of the CVB3-infected mice resulted in an earlier response of IFN-γ in the brain, i.e. 4 of 6 As_2O_3-treated mice were positive for IFN-γ already on day 5 of the study, whereas the untreated mice were not positive until day 7 of the infection (5 of 6 mice). The results of the untreated mice were com-

parable with a previous study using the same experimental CVB3 model, where IFN-γ peaked on day 6 in serum (110). IFN-γ treatment powerfully reduced titres of vesicular stomatitis virus, influenza A virus, HSV-1 and measles virus in infected neuronal cells *in vitro*, demonstrating its potent antiviral activity (96, 131). Horwitz et al. showed that transgenic mice expressing IFN-γ in the pancreas failed to develop CVB3-induced myocarditis, probably by reducing the viremia already at an early stage of the infection (69). Thus, the As_2O_3-induced response in IFN-γ in the present study likely contributed to the antiviral effect of As_2O_3 in the brain. However, As_2O_3 treatment had no effect on IFN-γ in the liver and pancreas in the present study, indicating a tissue-specific effect of As_2O_3 on IFN-γ.

It has been reported that the promyelocytic leukaemia protein, a target for As_2O_3 therapy during this type of leukaemia, is also involved in the antiviral defence (117). It is further believed that IFN-γ may increase the expression of this protein (28). As_2O_3-induced apoptosis in APL cells was enhanced by simultaneous addition of IFN-γ (27). On the other hand, IFN-γ was recently suggested to play a protective role in sodium arsenite-induced renal injury by up-regulating the expression of intrarenal multidrug resistance-associated protein 1 (91). Furthermore, a difference in the expression of this resistance protein was the suggested explanation for strain differences among mice in As tolerance (92). Thus, As_2O_3-enhanced gene expression of IFN-γ in the brain of the infected mice may have an antiviral effect, a detoxifying mechanism for As, or both.

Concluding remarks and future perspectives

The initial papers in this study showed that both essential and non-essential trace elements are affected in serum and tissues during the common viral infection of CVB3 in mice. The most consistent changes in trace elements were found in Se and As (paper I) that both decreased in most of the studied tissues. Others have shown that a viral infection in a host deficient in Se not only may aggravate the clinical course of the infection (9, 10) but also affect the genomics of the virus particle by creating an opportunity for mutation into more virulent forms (12). The present study indicated that not only Se but also As could be involved in the host defence against CVB3 infections.

Administration of a clinically relevant dose of As_2O_3 to healthy mice resulted in minor changes in trace elements in serum and tissues (paper II). This was a surprising finding because As has been found to affect both Se and Zn, two trace elements essential for the immune system (55, 100, 104). This finding is not consistent with findings when the same dose of As_2O_3 was administered to CVB3-infected mice. Thus, in infected mice administration of As_2O_3 resulted in more pronounced effects on several trace elements when compared with non-infected mice, with an increase in Zn and As, but a decrease in Se in most tissues (paper III). The reduction in Se recorded in the infected, As_2O_3-treated mice (paper III) was comparable with the reduction in Se seen during an acute CVB3 infection in untreated mice (paper I) and was thus probably a result of the infection *per se*, rather than an effect of the treatment. It therefore seemed reasonable to conclude that As *per se* is involved in the immune processes, directly in the viral replication, or both.

Daily As_2O_3 treatment during 7 days of an acute CVB3 infection reduced the viral RNA load in the pancreas and brain, two of the target organs of this infection (paper IV). This antiviral effect of As in the brain is noteworthy since many therapeutic drugs do not efficiently penetrate the BBB and are thus unable to reach therapeutic concentrations in the brain (130). Furthermore, the *in vitro* experiment in this study indicated that the antiviral effect of As_2O_3 was a result of a disturbed virion assembly or release of virus from the infected cells, which would result in a decreased number of virus particles available for re-infection of new cells (paper IV).

To investigate whether As_2O_3 is also associated with general immune-mediated mechanisms that potentially could affect viral load the effects of As_2O_3 on NFκB and IFN-γ were studied during a CVB3 infection in mice. However, As_2O_3 treatment for 7 days was found to only affect the infection-

induced expression of IFN-γ in the brain, where the treatment activated IFN-γ on day 5 of the infection instead of day 7 as in the untreated mice.

Based on the observed decrease in As concentration in target organs of the present CVB3 infection, together with findings of a direct antiviral effect of As, it can be hypothesised that As could be a novel candidate in the future development of antiviral therapy, either alone or in combination with other drugs. However, before As can be implemented in clinical studies on viral diseases, a thorough safety evaluation of potential toxic effects of As treatment, together with mechanistic studies on replication and release of virus, effects on immune cells and metabolic pathways is needed.

In the present study the effects of As_2O_3 on viral replication were measured as changes in viral nucleic acid concentration. However, nucleic acid is not the same thing as viable viral particles. Thus, a future step would be to culture viral particles from culture medium as well as from the lysed cells to investigate whether the virus is infectious after As_2O_3 treatment. Electron microscopy studies on As distribution in the cells and full-genome sequencing of the viral particle would also be of interest. As_2O_3 treatment could possibly change the viral genome sequence, as was the case when the CVB3 genome sequence was changed by mutation during infection in Se-deficient mice (12).

Myocarditis and inflammatory or dilated cardiomyopathy are both possible complications associated with CVB3 infection that can occur weeks (myocarditis) to years (inflammatory or dilated cardiomyopathy) after a viral infection (89). Reetoo et al. found viral RNA in murine hearts months after the active infection (138). Thus, the immune system may evidently fail to eradicate the virus, suggesting it could cause sustained immune stimulation resulting in late phase inflammatory cardiomyopathy. Moreover, presently there are no antiviral drugs in the treatment of EV infections on the European market. A possible extension of the present studies would be to use a combined therapy with a drug with immunomodulating or anti-inflammatory action together with the use of As_2O_3, presumably impairing viral replication.

In conclusion, this thesis shows that a clinically relevant dose of As_2O_3 reduces replication of CVB3 in the mouse brain and pancreas, as well as in infected cell culture. As_2O_3 treatment did not substantially affect the balance of other trace elements; nor general immune-mediated mechanisms, except in the brain. The antiviral effect of As_2O_3 is thus likely to be a direct effect on viral replication or viral release from infected cells. Although the mechanisms underlying the observed effects on viral replication remain to be elucidated, As is an intriguing trace element to study in the pursuit of new, efficient antiviral drugs.

Sammanfattning på svenska

Spårelement är grundämnen som i små mängder finns i kroppen. Flera spårelement, till exempel zink, järn, koppar och selen är livsnödvändiga (essentiella) för ett väl fungerande immunförsvar och koncentrationen av dessa spårelement förändras i kroppen under en infektion. Tillskott av det essentiella spårelementet selen har i mus visat sig kunna begränsa en coxsackievirus B3-infektion. Ett fåtal studier tyder på att även det icke-essentiella spårelementet arsenik, som finns i kroppen i små mängder men som inte är nödvändigt för kroppens funktioner, kan påverka produktionen av vissa virus.

I **delarbete I** undersöktes hur balansen av essentiella och icke-essentiella spårelement i kroppen påverkas av en vanlig virusinfektion (coxsackievirus B3) i mus. Studien visade att koncentrationen av selen minskade kraftigt i nästan alla infekterade vävnader, och även att selenkoncentrationen var negativt korrelerad till virusmängden i vissa vävnader, alltså att mer selen var förenat med mindre virus och vice versa. Dessutom minskade koncentrationen av arsenik kraftigt i nästan alla infekterade vävnader. Andra forskargrupper har visat att brist på selen kan förvärra infektionsförloppet under en virusinfektion. Denna studie indikerade att inte bara selen, utan även arsenik kan vara inblandat i kroppens försvar eller vara viktigt för mängden virus som bildas under en coxsackievirus B3-infektion.

Arsenik har visat sig kunna interagera med selen och zink, två för immunförsvaret essentiella spårelement. **Delarbete II** visade mot förmodan att en låg dos av arsenik i friska möss påverkade balansen av andra spårelement förvånansvärt lite. Trots att koncentrationen av arsenik i olika vävnader ökade markant på grund av arseniktillskottet så var förändringarna i zink och selen marginella. **Delarbete III** visade däremot att arseniktillskott under pågående coxsackievirus B3-infektion påverkade balansen av spårelement olika i friska och sjuka möss, med större effekter i de infekterade djuren. Bland annat resulterade arseniktillskottet i högre arsenikkoncentration i vävnaderna i infekterade jämfört med friska möss, trots att de fått samma dos arsenik. Slutsatsen från ovanstående tre delarbeten var därför att arsenik är direkt involverat i immunförsvaret eller i mekanismerna för virusförökning.

Delarbete IV visade att sju dagars behandling av coxsackievirus B3-infekterade möss med en låg dos av arsenik minskade mängden virus i hjärnan och bukspottkörteln, två av målorganen vid denna infektion, i vilka det i vissa fall utvecklas svåra komplikationer. Att virusmängden i hjärnan minskade är ett spännande fynd eftersom många läkemedel har svårt att passera

genom den skyddande barriär som omger hjärnan och dess hinnor och därigenom nå fram till viruset. I detta delarbete gjordes även cellförsök som tyder på att arsenik troligtvis bromsar virusinfektionen genom att hämma frisättandet av nybildade viruspartiklar från en infekterad cell. På så sätt skulle arsenik kunna förhindra att viruset infekterar nya friska celler och därmed sprids i kroppen. **Delarbete V** visade att arseniktillskott till infekterade djur inte hade någon entydig effekt på delar av det immun-medierade värdförsvaret som omfattas av nuclear factor κB (NFκB) och interferon γ (IFN-γ). Den virus-hämmande effekten av arsenik är alltså sannolikt en direkt effekt på virus och dess funktion.

Sammanfattningsvis visar avhandlingen att en låg och troligen kliniskt användbar dos av arsenik minskar virusmängderna i två viktiga målorgan för en coxsackievirus B3-infektion i mus, och att arsenik även minskar virusmängderna i cellkultur. Den använda dosen av arsenik störde inte heller nämnvärt balansen av essentiella spårelement i kroppen och påverkade inte heller immun-medierade mekanismer viktiga för infektionsförsvaret. Arsenik är alltså ett mycket intressant spårelement för fortsatta studier i syfte att utveckla ett nytt antiviralt läkemedel.

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