# COXSACKIEVIRUS INFECTION OF MICE. I. VIRAL KINETICS AND HISTOPATHOLOGICAL CHANGES IN MICE EXPERIMENTALLY INFECTED WITH COXSACKIEVIRUSES B3 AND B4 BY ORAL ROUTE

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**Summary. –** We followed the viral kinetics and histopathological changes in different organs of immunocompetent mice infected orally with coxsackieviruses B3 (CVB3) Nancy strain and B4 (CVB4) JVB strain separately. The viruses used were not adapted to mouse organs. In the acute phase of infection, the viral kinetics indicated virus replication in the heart, spleen, thymus, pancreas, and small and large intestines. This was accompanied by histopathological changes, mild infiltration of mononuclear cells and fibrosis in the heart. The necrotic changes with mononuclear infiltration and fibrosis in the myocard was observed on days 56 and 71 p.i. in the CVB4-infected animals only. In the mice infected with CVB3 and CVB4 a prolonged presence of infectious virus was shown in the spleen and small intestine; in the latter viral antigen was localized in smooth muscles of the muscular wall immunohistochemically. This is the first report on prolonged replication of coxsackieviruses (CV) in the spleen and small intestine in orally infected mice.

**Key words:** Coxsackie B3 virus; Coxsackie B4 virus; mouse; oral infection; viral kinetics; histopathology

## Introduction

A broad spectrum of diseases is associated with CV reflecting their wide tissue tropism and differences in virulence. Over the last decades CVB have been recognized to be associated with acute myocarditis, endocarditis and pericarditis. In addition to established associations with acute diseases, CVB infection has been implicated in several chronic diseases, such as diabetes mellitus or chronic heart diseases (reviewed by Muir *et al*., 1989; Hyoty *et al*., 1998; Jun and Yoon, 2001; Kim *et al.*, 2001).

Several variables, such as host genetics, immunological status, sex and age, as well as viral genetics have been shown to be involved in CVB damage to target organs, and thus to influence the outcome of infection (Herskowitz *et al*., 1987; Huber and Pfaeffle, 1994; Huber, 1997).

CVB replicate in murine host and induce diseases, which mimic those in humans, and thus the mouse model is useful for the study of enteroviral pathogenesis (reviewed by Tracy *et al*., 2000). For studying the pathogenesis inbred, semi-inbred, knockout, transgenic and nude athymic mice have been often used (Gauntt *et al*., 1984; Mena *et al*., 2000; Schwimmbeck *et al*., 2000; Tracy *et al*., 2000; Huber *et al*., 2001; Kishimoto *et al*., 2001). The outbred model, though very seldom used (Merkle *et al*., 1999; Glück *et al*., 2001) in comparison with the inbred model, represents the natural

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**Abbreviations:** CV = coxsackieviruses; CVB = Coxsackievirus B; CPE = cytopathic effect; GALT = gut-associated lymphoid tissue; i.p. = intraperitoneal; IU = international unit; MAb = monoclonal antibody; PCR = polymerase chain reaction; PBS = phosphate-buffered saline; p.i. = post infection; VP = viral protein

variation in human population. Most of the studies on CVB3 experimental murine models make use of intraperitoneal (i.p.) route of infection; however, the natural route of infection in humans is the fecal-oral one. Oral infection with CVB1, CVB3 and CVB5 in neonatal, adult and pregnant mice has been reported earlier (Kaplan and Melnick, 1951; Loria *et al*., 1974 a,b, 1977; Petrovičová, 1983; Modlin and Bowman 1987; Bourlet *et al*., 1997), documenting a dose-dependent mild infection in adult mice.

Enteroviruses show a high degree of variation of genome nucleotide sequence and a high frequence of recombination events which occur both between and within serotypes and might affect their tissue tropism (Lindberg *et al*., 2003). Pathogenesis and the role of CV in the development of chronic diseases are too complex to understand mainly due to the variation in the genetics of both the host and the virus inclusive of immunological background of the host. A close genetic relationship of six CVB serotypes is known (Hyypia *et al*., 1997; Romero *et al*., 1997)

The aim of the present study was to follow the virus kinetics, histopathological changes and antibody response in immunocompetent outbred mice infected perorally with two different serotypes of CV, namely B3 and B4, over a prolonged time period. We used CVB3 Nancy strain and CVB4 JVB strain, which were not adapted to specific tissues such as the brain, heart or pancreas.

## Materials and Methods

*Cells and viruses*. The strains CVB3 Nancy and CVB4 JVB were obtained from National Institute of Health, Prague, Czech Republic, was propagated in green monkey kidney (GMK) cells. For virus isolation and titration Hep-2 cells were used. Both the cell lines were grown in Leibovitz (L15) medium supplemented with 5% or 2% bovine serum.

*Mice*. Three-to-four week-old Swiss albino mice (ICR strain) weighing 10–17 g were obtained from the Velaz Farm, Prague, Czech Republic.

*Oral infection*. Mice were infected by oral gavage, using a sterile polyethelene tube and syringe, with 5 x $10^9$ $TCID_{50}$ of either virus in 0.5 ml of phospate-buffered saline (PBS). Negative (uninfected) control mice obtained 0.5 ml of PBS only. Infected and control mice were sacrificed daily from day 0 to day 10 post infection (p.i.) and then weekly from days 14 to 63 and day 71 p.i. (CVB4) and day 98 p.i. (CVB3). The blood was obtained by cardiac puncture, and portions of the heart, pancreas, thymus, spleen, and small and large intestines were washed in PBS and either snap frozen in liquid nitrogen and stored at -80ºC or fixed in 10% formaldehyde for histological studies.

*Virus isolation in cell cultures.* Snap-frozen tissues were freeze-thawed twice and 10% suspensions in PBS were prepared by

**Table 1. Titers of CVB3 and CVB4 antibodies in sera of orally infected mice**

| Days p.i. | Control | Infected | |
|---|---|---|---|
| | | CVB3 | CVB4 |
| 0 | <4 | <4 | <4 |
| 3 | ND | <4 | <4 |
| 5 | <4 | <4 | 4 |
| 7 | ND | 128 | 256 |
| 10 | ND | 1024 | 64 |
| 14 | ND | 256 | 32 |
| 21 | <4 | 256 | 32 |
| 28 | ND | 128 | 8 |
| 35 | ND | 128 | 16 |
| 49 | <4 | 64 | 16 |
| 63 | ND | 32 | 4 |
| 98 | <4 | 32 | 4 |

**Table 2. Viral load ($log_{10}$ $TCID_{50}$/ml) in organs of mice orally infected with CVB3 Nancy strain**

| Organ | Day p.i. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 3 | 5 | 7 | 10 | 14 | 21 | 28 | 35 | 49 |
| Heart | – | 2.50 | 2.50 | 3.4667 | 1.0 | 1.5 | + | – | – | – |
| (CI) | | (0.5658) | 0.5567 | (0.6232) | (0.5092 | 0.5657 | | | | |
| Pancreas | – | 2.0173 | 1.45 | 2.485 | 1.35 | 1.5333 | – | – | – | – |
| (CI) | | (0.5377) | (0.4490) | (1.1901) | (0.6376) | 0.5667 | | | | |
| Thymus | – | + | + | 1.0167 | +++ | 1.5 | – | – | – | – |
| (CI) | | | | (0.7598) | | (1.1316) | | | | |
| Spleen | – | 1.5 | 1.5 | 2.5007 | 2.0167 | 1.5 | 1.0167 | 1.0 | 1.0333 | – |
| (CI) | | (0.5658) | (0.4899) | (1.1620) | (1.1447) | (1.1609) | (1.3115) | (0.5658) | (0.3315) | |
| S. intestine | – | 1.515 | 1.1667 | 1.5333 | – | – | – | + | 1.0423 | +++ |
| (CI) | | (0.820) | 0.5943 | (0.6797) | | | | | (1.2057) | |
| L. intestine | – | 1.135 | +++ | 1,5067 | – | – | – | – | – | – |
| (CI) | | (0.5699) | | (0.5594) | | | | | | |

(–) = no infectious virus.
Infectious virus present in pool No. 1 (+), No. 2 (++) or No. 3 (+++). Each pool consisted of parts of three organs.
CI = confidence interval.

sonication 30 mins in a Soni Dismemberator (Dynatech Fischer) at 50% intensity for 1 min followed by centrifugation at 1,500 x g for 20 mins at 4°C. At this point 200 U/ml penicillin and 200 µg/ml streptomycin were added. In the case of small and large intestines, tissues were processed similarly except that after centrifugation the supernatant was shaken with equal volume of 1,1,2-trichlorotrifluro-ethane by vortexing for 3 mins, centrifuged at 1,500 x g for 20 mins, the upper liquid phase was collected, and 200 U/ml penicillin, 200 µg/ml streptomycin and 100 µg/ml gentamycin were added. The suspensions were incubated overnight at 4°C, centrifuged at 1,500 x g for 30 min at 4°C, passed through a 0.45 µm Millipore filter and frozen at -80°C. Pooled homogenates from three animals were used for virus isolations and titrations.

*Titration of infectious virus in organs*. In order to infect monolayers of Hep-2 cells in 96-well flat bottom microtiter plates and to incubate them at 37°C in a $CO_2$ incubator tenfold dilutions of the sera and organ suspensions were prepared. Results were read on day 4 p.i. Virus titers were expressed as $TCID_{50}$, calculated in a standard way.

*Virus-neutralizing antibody titers* of sera were determined on the basis of inhibition of cytopathic effect (CPE) in a standard way (Gauntt *et al*., 1979). Twofold dilutions (1:4 to 1:1024) of sera were used. CPE was evaluated on the day 7 of incubation. The titer represented the reciprocal of the serum dilution ensuring 50% protection against CPE.

*Histology*. Serial 4–7 µm thick sections of formalin-fixed and paraffin-embedded samples of the heart, spleen, pancreas and small intestine were stained with hematoxylin and eosin in a standard way. Cellular infiltration (I) and necrosis (N) in the heart tissue was graded as described by Opavsky *et al*. (1999). A score of 0 corresponded to the absence of inflammation or necrosis; 1 incipient, focal, I and N; 2 mild to moderate, I and N; 3 moderate, I and N; 4 extensive areas of I and N involving a large part of the examined heart tissue. The pancreas and small intestine were also examined for histological changes.

*Localization of antigen in the small intestine by immunohistochemical staining*. The specimens for immunohistochemical staining were heated three times for 5 mins in a 10 mmol/l citrate buffer in a microwave oven. The sections were then cooled, immersed in 3% $H_2O_2$ for 20 mins to inhibit endogenous peroxidase activity. A mouse monoclonal antibody (MAb) against the enterovirus VP1 (clone 5-D8/Isotype Ig2a Kappa, DAKO), used as the primary antibody, was diluted 1:200. For the immunostaining the Animal Research Kit (DAKO) was used. This method makes use of a biotinylated secondary antibody, which binds to the primary antibody thus minimizing potential interaction with endogenous immunoglobulin in the specimen. The method was modified and standardized by using a serum-free protein block (DAKO). The sections were counterstained with hematoxylin and examined microscopically. Brown staining reflected positivity, i.e. the presence of the enteroviral antigen.

*Statistical analysis*. Pools of 3 organs (n = 3) and a 95% confidence interval were used.

## Results

### *Serum antibody response to infection*

As seen in Table 1, neutralizing antibody titers in the sera of infected mice were detectable first at days 5 and 7 p.i. for CVB4 and CVB3, respectively, and remained detectable up to the end of the experiment. CVB3 led to higher antibody titers compared to CVB4. Control mice showed no antibody response.

### *Kinetics of peroral CVB3 and CVB4 infections*

Tables 2 and 3 show the virus kinetics in the organs of CVB3- and CVB4-infected mice, respectively. An increase in the virus titer in the heart of CVB3-infected mice from day 3 to day 7 p.i (with maximum of $10^{3.5}$ $TCID_{50}$/ml) and a subsequent decrease were observed. On the other hand the

**Table 3. Viral load ($log_{10}$ $TCID_{50}$/ml) in organs of mice infected orally with CVB4 JVB strain**

| Organ | Day p.i. | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 3 | 5 | 7 | 10 | 14 | 21 | 28 | 35 | 49 | 56 |
| Heart | – | – | – | 1.21 | +++ | +++ | – | – | – | – | – |
| (CI) | | | | (0.324) | | | | | | | |
| Pancreas | – | – | 1.02 | 1.44 | +++ | – | – | – | – | – | – |
| (CI) | | | (0.343) | (1.293) | | | | | | | |
| Thymus | – | – | +++ | +++ | +++ | – | – | – | – | – | – |
| (CI) | | | | | | | | | | | |
| Spleen | – | – | ++ | 1.46 | +++ | +++ | 2.46 | 1.01 | 1.01 | – | – |
| (CI) | | | | (1.310) | | | (0.912) | (0.014) | (0.718) | | |
| S. intestine | – | 2.29 | +++ | +++ | – | – | – | – | – | +++ | +++ |
| (CI) | | (1.177) | | | | | | | | | |
| L. intestine | – | 1.54 | +++ | 1.51 | – | – | – | – | – | – | – |
| (CI) | | (0.793) | | (0.826) | | | | | | | |

(–) = no infectious virus.
Infectious virus present in pool No. 1 (+), No. 2 (++) or No. 3 (+++). Each pool consisted of parts of three organs.
CI = confidence interval.

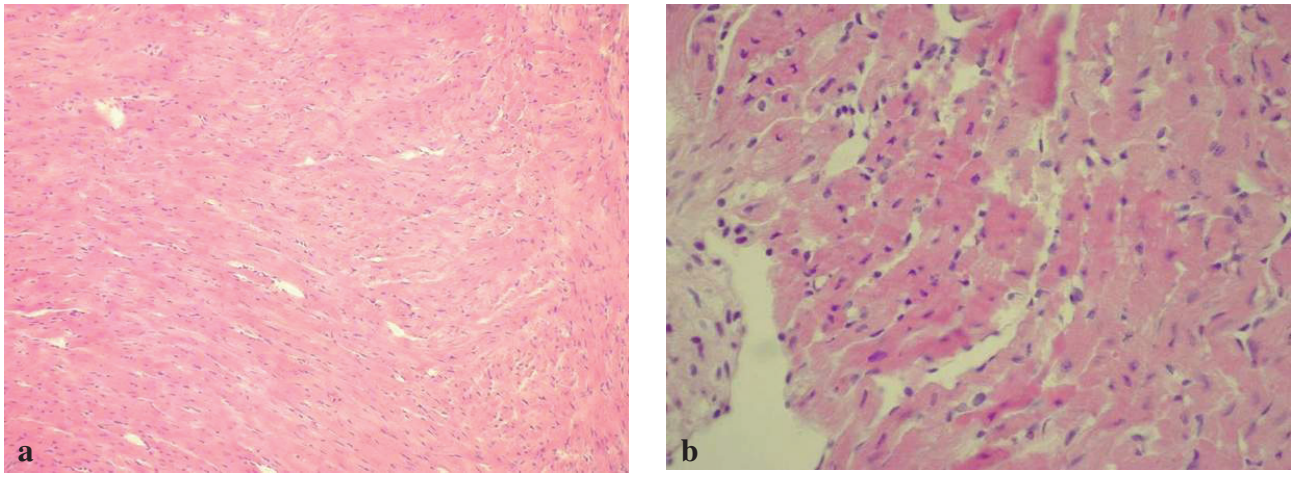


**Fig. 1**
**Heart sections stained with hematoxylin-eosin**
Mock-infected mouse (a). CVB3-infected mouse, day 10 p.i. (b). Magnification 250x.

heart of CVB4-infected mice showed a maximum titer of $10^{1.2}$ $TCID_{50}$/ml on day 10 p.i.

The pancreas of the infected mice showed a rise of the virus titer up to $10^{2.5}$ $TCID_{50}$/ml (CVB3) and $10^{1.4}$ $TCID_{50}$/ml (CV B4) by day 7 p.i. and a slow decrease leading up to absence of the virus on day 21 p.i. and later intervals.

In the thymus CVB3 was detectable from day 3 to day 14 p.i. at a titer of $10^{1.5}$ $TCID_{50}$/ml while CVB4 was present at very low levels only from day 5 to day 10 p.i.

In the spleen both CVB3 and CVB4 could be detected from day 3 until day 35 p.i. with a peak on day 7 p.i. ($10^{2.5}$ $TCID_{50}$/ml and $10^{1.5}$ $TCID_{50}$/ml, respectively), followed by a slow decrease to day 35 p.i.

In the large intestine the virus was detectable only for a short time period, from day 3 to day 7 p.i. with maximum titers of $10^{1.5}$ $TCID_{50}$/ml for both viruses.

*Macroscopic observations*

The small intestine of the mice infected with CVB3 or CVB4 were on day 28 or 56 p.i., respectively, pink and inflamed and the consistency of the excreta in those parts of concern was highly mucoid. However, as the final consistency of the excreta returned to normal the emphasis was given to the virological and histological observations.

*Histological observations*

In the heart of mice infected with either CVB3 or CVB4 small foci of necrosis and a moderately increased number of mononuclear cells (grade 2) were observed on day 10 p.i.; the necrotic foci resolved by day 28 p.i. (Fig. 1). These changes were not present in the control. Infiltration of necrotic myocardial cells with mononuclear and fat cells was observed at grade 3 in a CVB4-infected mouse showing a very large focus on days 56 and 71 p.i.; this change was absent in the control (Fig. 2).

No pathological changes were seen in the endocrine or exocrine pancreas of infected mice.

In the small intestines of infected mice enlarged Peyer's patches accompanied by degenerative changes were observed in the villi just beneath the Peyer's patches on day 3 p.i. though the lining of the columnar epithelium was well preserved. Mock-infected control mice showed normal villi and a well preserved lining of columnar epithelium.

Histopathological examination of CVB3-infected mice on day 28 p.i. revealed a severe diffuse degeneration of the gut epithelium, inflammatory changes and destruction of villi in apical parts and considerable amount of cell debris in the intestinal lumen. Similar changes were observed in the mucosa and submucosa in the lining of the small intestines of the CVB4-infected mice on day 56 p.i.

*Immunohistochemical analysis of viral antigen in the small intestine*

In the CVB3- and CVB4-infected mice on days 28 and 56 p.i., when macroscopic changes could be seen, the viral antigen was observed in smooth muscles of the small intestines (Fig. 3b and 3c, respectively). Mock-infected control mice showed no viral antigen in the small intestines (Fig. 3a).

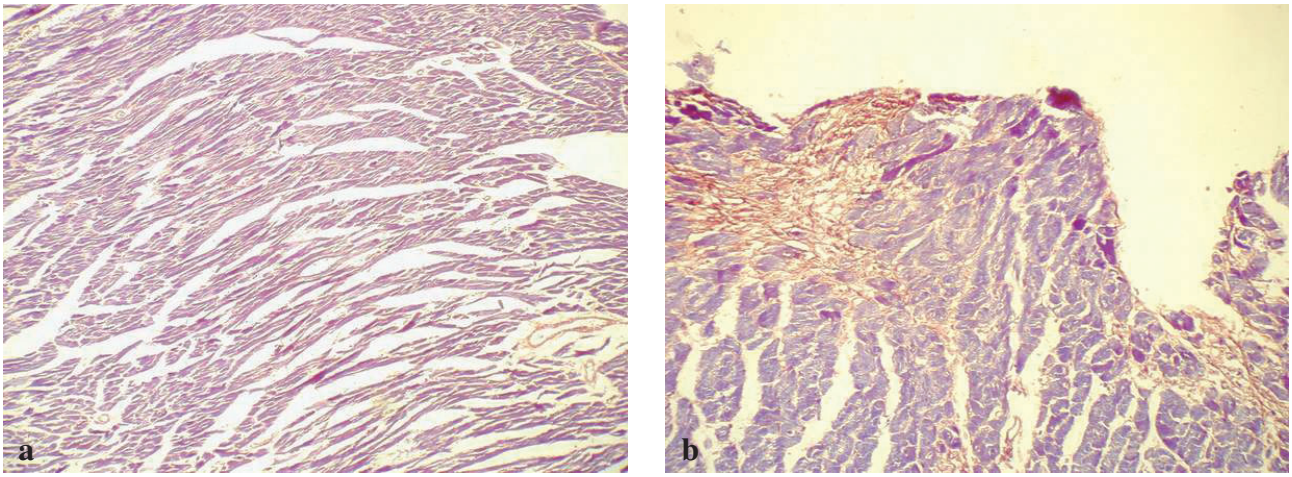


**Fig. 2**
**Heart sections stained by the Malorie's method**
Mock-infected mouse (a). CVB4-infected mouse, day 71 p.i. (b). Magnification 200x.

## Discussion

The natural route of transmission of enteroviruses is fecal-oral. Hence an oral model of infection would be useful and relevant for studying the pathogenesis of entroviruses. In this respect polioviruses are the most studied enteroviruses; oral infection with poliovirus has been so far studied in monkeys (Kanamitsu, 1967) and in TgPVR transgenic mice carrying the human poliovirus receptor (Zhang and Racaniello, 1997). The mechanisms by which poliovirus spreads from the gut has not yet been completely characterized. Studies on oral route of infection of CVB have been limited to a short time period, namely to days 14–21 p.i. (Kaplan and Melnick, 1951; Loria et al., 1974 a,b, 1977; Petrovičová, 1983; Modlin and Bowman, 1987; Bourlet et al., 1997).

Loria *et al.* (1977) have found a 4- to 5-fold reduction in the CVB titer in adult mice after encountering the gut-associated lymphoid tissue (GALT) barrier. GALT contains lymphocytes, granulocytes, macrophages, plasma cells, and dendritic cells; immunoblasts, i.e. transformed lymphocytes should play a role in local immunity (reviewed by

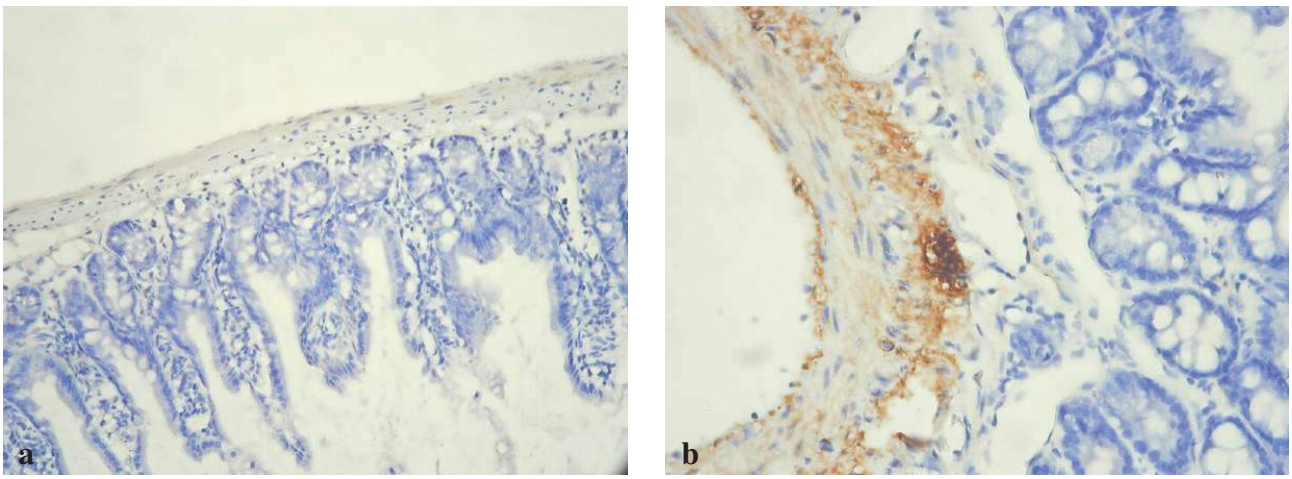


**Fig. 3**
**Localization of CV capsid protein VP1 in sections of the small intestine by use of immunohistochemical staining**
Mock-infected mouse (a). CVB3-infected mouse, day 28 p.i. (b). CVB4-infected mouse, day 56 p.i. Magnification 200x.

Brandtzaeg, 1998; Ogra *et al.*, 2001). This phenomenon has been observed by Loria *et al*. (1974a) in newborn CD-1 mice, which showed a lower rate of paralysis and death after peroral infection with a small virus dose ($\leq 10^2$ PFU) as compared to that resulting from a large virus dose ($10^6$ PFU). In our preliminary study (Petrovičová, unpublished results), a successful peroral infection of 4-week-old Swiss albino mice with $10^{6.5}$ $LD_{50}$ of CVB1 Conn-5 strain adapted to suckling mice has been achieved. These facts justify the application of a high oral dose of 5 x $10^9$ PFU of CVB3 Nancy strain and CVB4 JVB strain in the present study.

The viral kinetics as determined in the present study implies that the viral dissemination to numerous target organs following the oral route of infection was successfully initiated. Neither morbidity nor mortality were observed in the infected mice. Of various organs tested it was the heart and spleen, which showed the highest virus titers.

Infectious virus was observed in all organs showing systemic infection. This observation is consistent with that of Loria (1974b) regarding oral infection of adult mice with CVB5 the mice were susceptible to infection in the absence of outward clinical manifestations. Our results differ from those obtained by other authors on mice infected with poliovirus by oral route (Zhang and Racniello, 1997). In the latter study poliovirus did not replicate in the intestines of the TgPVR transgenic mice and was not disseminated either. These differences may be related to entirely different hosts and viruses, even though CV and polioviruses belong to the same genus, *Enterovirus*.

Data demonstrating genetic differences in mouse strains defining the extent and duration of CVB3 viremia, appearance of neutralizing antibody titers (Wolfgram *et al*., 1986) and multigenetic control of early and late histopatological changes in the case of i.p. route of infection have been shown by Herkowitz *et al*. (1987). Our data on virus kinetics and heart histopathology in the acute phase of infection are conform with them. Virus replication in pancreatic tissue is not an indicator of pancreato- or cardiovirulent phenotype since even within one serotype different strains may show differences in virulence (Tracy *et al*., 2000). Absence of histological changes of exocrine and endocrine pancreatic cells in our experiments in the acute phase of infection with CVB3 may be a consequence of oral infection or the fact that the virus strain used by us was not pancreatovirulent.

Our observation that the small intestines of orally infected mice showed degenerative changes in the villi on day 3 p.i. just beneath the Peyer patch is not conform with that of Loria *et al*. (1977), who have showed lack of inflammatory changes during first 3 day p.i. in adult CD-1 (semi-inbred) mice infected with CVB5. Our observation may be limited to the CVB strain used and a consequence of the oral mode of infection. Anderson *et al*. (1996) have shown a temporal load of infectious virus in the spleen of A/J and C57/BL/6J mice during the early stage of CVB3 infection and *in vitro* replication in the splenocytes from uninfected mice thus indicating an association of CVB3 with splenocytes. We have shown a prolonged presence of infectious virus in the small intestines and such virus persistence in the gastrointestinal tract of agammaglobulinemic patients, from where it may invade the central nervous system, had been predicted (Galama, 1997).

The defense against any pathogen is initiated at the portal of entry and this may affect the course of infection, virus dissemination and pathogenesis (Resigno and Borrow, 2001). We suggest that the prolonged presence of infectious virus and absence of histolpathological changes in the pancreas are a consequence of the oral route of infection. A sufficient number of CVB murine models have been generated, but for a model careful selection of virus variant, mouse strain, age of the mouse and the time of virus challenge and to a lesser extent gender of the mouse must be selected (Gauntt *et al*., 1993). Therefore, to characterize the oral route of infection completely, a few questions arising from the present study should be answered: (i) is a high virus dose essential for initiation of systemic infection and prolonged presence of the virus in the experimental oral mouse model? (ii) would a sensitive method such as PCR detect viral RNA provided infectious virus was not detected by a classical tissue culture method? A study motivated by these questions is underway.

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