Coxsackie B virus infection of mice: inoculation by the oral route protects the pancreas from damage, but not from infection

Shubhada Bopegamage,1 Jana Kovacova,1 Agnesa Vargova,1 Jana Motusova,1 Anna Petrovicova,1 Maria Benkovicova,2 Pavol Gomolcak,2 Judith Bakkers,3 Frank van Kuppeveld,3 Willem J. G. Melchers3 and Jochem M. Galama3

Correspondence
Jochem M. Galama
j.galama@mmb.umcn.nl

1Department of Virology, Slovak Medical University, Limbova 12, 83303 Bratislava, Slovak Republic
2Institute of Pathology, Derer’s Hospital and Clinic, Limbova 5, 83301 Bratislava, Slovak Republic
3Virology Section, Department of Medical Microbiology, Radboud University Medical Center Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands

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The pathogenesis of coxsackie B virus (CVB) infections is generally studied in mice by intraperitoneal (i.p.) injection, whereas the gastrointestinal tract is the natural porte d’entrée in humans. The present study was undertaken to compare systematically the influence of infection route on morbidity and pathology. Swiss Albino mice were infected with CVB3 (Nancy) at different doses (5 \times 10^3, 5 \times 10^5, 5 \times 10^7, 5 \times 10^9 TCID\text{50}) , given either i.p. or orally. Virus could be isolated from several organs (heart, spleen and pancreas), indicating systemic infection, irrespective of the infection route. Virus titres were 1–2 logs higher after i.p. infection, but kinetics were largely independent of infection route. Organs became negative for virus isolation after 21 days, with the exception of spleen tissue, which remained positive for up to 49 days. Thereafter, virus was detected only by immunohistochemistry and PCR up to 98 days post-infection (oral route). Histopathology showed mild inflammation and necrosis in heart tissue of all mice during the acute phase, with repair at later stages. Strikingly, pancreatic lesions were confined to the exocrine pancreas and observed only after i.p. infection. Under all experimental conditions, the pancreatic islets were spared. In contrast, immunohistochemistry showed the presence of viral VP1, protein 3A and alpha interferon (IFN-\text{a}) in exocrine as well as endocrine pancreas of all mice, irrespective of route and dose of infection. It is concluded that infection via the oral route protects the pancreas from damage, but not from infection, a process in which IFN-\text{a} is not the only factor involved.

INTRODUCTION

Coxsackieviruses (CVBs) belong to the genus Enterovirus of the family Picornaviridae, which is a large and complex family of small RNA viruses with a genome of positive polarity. The human enteroviruses (HEVs) are classified into five genetic types: Poliovirus and HEV-A, -B, -C and -D (King et al., 2000). CVBs, six serotypes of which are known (CVB1–CVB6), belong to the HEV-B type. In man, HEVs are transmitted mainly by the faecal–oral route, but respiratory transmission may also occur (Pallansch & Roos, 2001). Infections by HEV-B are highly prevalent, but they are usually subclinical or cause a mild flu-like illness. However, HEV infections can be serious, with manifestations of meningo-encephalitis, paralysis, myocarditis and, in neonates, a fulminant sepsis-like syndrome (Galama, 1997; Pallansch & Roos, 2001). Sporadically, a fulminant HEV-B infection can result in acute onset of type 1 diabetes without autoimmunity (Vreugdenhil et al., 2000; Yoon et al., 1979). These viruses are furthermore incriminated as causes of chronic (inflammatory) diseases such as chronic myocarditis (Gauntt, 2003), chronic pancreatitis (Ramsingh et al., 1997) and the more common autoimmune type 1 diabetes (Hyöty & Taylor, 2002). Whether these chronic diseases are related to virus persistence is still a point of discussion (Melchers et al., 1994). Hence, the pathogenesis of CVB infections is complex and, as yet, far from clear.

Coxsackieviruses also cause infection in mice; thus, mouse models have been established to study the pathogenesis of CVBs, in particular their putative role in myositis, chronic myocarditis, pancreatitis and type 1 diabetes (reviewed by...
Gauntt, 2003; Horwitz et al., 1998; Ramsingh et al., 1997; See & Tilles, 1995; Tracy et al., 2002). Most of the mouse studies carried out so far have used the intraperitoneal (i.p.) route of infection, which is mainly because this infection route causes consistently more morbidity and even mortality than the oral infection route.

Oral infection with CVB1, CVB3 or CVB5 in neonatal, adult and pregnant mice has been reported previously (Bourlet et al., 1997; Harrath et al., 2004; Kaplan & Melnick, 1951; Loria et al., 1974a, b, 1976; Modlin & Bowman, 1987; Petrovicova, 1983) and with enterovirus 71 (Chen et al., 2004). In contrast to neonatal mice, adult mice show a very mild outcome of infection, from which it was postulated that some intrinsic immunity matured in the gut of adult mice, which hampered viraemia and moderated the course of infection (Loria et al., 1976). This observation has a marked resemblance to the clinical course in humans, where severe infections occur mainly in the neonatal period and mild infections occur at later ages. In spite of the similarity to infection in humans, Loria’s observation regarding the gut barrier has largely been overlooked, including in more recent studies that explored the oral route of infection (Bourlet et al., 1997; Chen et al., 2004; Harrath et al., 2004).

In the present work, the influence of the route of infection on CVB-induced pathology was studied. Previous studies with CVB3 (Nancy) and CVB4 (JVB) in Swiss Albino mice indicated systemic spread after oral infection with prolonged virus presence (98 days) in the small intestine, but it was unclear whether the high virus dose that was given orally influenced the outcome (Bopegamage et al., 2003). The questions that arose from these earlier studies were: (i) are there differences in pathology that can be attributed to the route of infection and are these differences related to the virus dose? Is, for instance, a high virus dose required for initiation of systemic infection via the oral route? (ii) Is persistent infection in the intestine and at other sites dependent on the route of infection? Here, a systematic comparison is presented between the oral and i.p. infection routes for outcome, regarding virus kinetics in blood, heart, pancreas, intestine and spleen, and for histopathology at the same time points. In addition, tissues were scrutinized by PCR for viral RNA and by immunohistochemistry for the presence and localization of virus protein 1 (VP1) and protein 3A, as well as expression of alpha interferon (IFN-α), which may be involved in the protective effect of infection by the oral route.

**METHODS**

**Virus and cells.** CVB3 (Nancy) was obtained from the National Institute of Health, Prague, Czech Republic, and propagated in Green monkey kidney (GMK) cells. HEp-2 cells were used for virus isolations and titrations. Cells were grown in Leibovitz (L15) medium supplemented with 5% heat-inactivated bovine serum for cell growth and 2% serum for maintenance and infection. For preparation of virus stocks, GMK cells were grown in Roux bottles and infected at an m.o.i. of 0-1. On day 3 post-infection (p.i.), cells were freeze-thawed three times and centrifuged at low speed for 10 min; supernatants were divided into aliquots and stored at −80°C. Virus stocks were titrated on GMK cells in 96-well microtitration plates by making tenfold dilutions (eight wells per dilution). Plates were incubated at 37°C in a CO2 incubator and the results were read daily until day 7 of incubation under the light microscope. Titres were expressed as TCID50 values, calculated according to Reed & Muench (1938).

**Mice.** Swiss Albino mice (ICR strain; 3–4 weeks old and 10–17 g) were acquired from Velaz Prague. All mice were rested for 1 week before inoculation. Mice were housed three per cage and supplied with sterile water and commercial food pellets (Topdovo). Permission for the animal work was obtained from the Ethics Committee of the Slovak Health University and the State Veterinary and Food Control Authority of the Slovak Republic.

**Oral infection.** The method utilized for oral infection of mice has been described previously (Bopegamage et al., 2003). Mice were divided into five groups of 33 mice. Each group received 0.5 ml virus suspension or PBS (uninfected controls) using a sterile polyethylene tube and a syringe. The following virus doses were used: 5×103, 5×104, 5×105 and 5×106 TCID50. Three infected and three control mice were sacrificed on days 3, 7, 10, 14, 21, 28, 35, 48, 56, 63, 98 and 147 p.i. Blood was obtained by cardiac puncture. Portions of heart, pancreas, thymus, spleen, and small and large intestines were obtained, washed in PBS and either snap-frozen in liquid nitrogen and stored at −80°C or fixed in 10% formaldehyde for histological studies.

**i.p. infection.** Mice were divided into different groups as described for oral infection and received the same virus doses (0.5 ml), but were injected i.p. Mice were sacrificed at the same time intervals and blood and organs were collected as described above for oral infection.

**Organ suspensions for virus isolations in tissue cultures.** Snap-frozen tissues were freeze-thawed twice and 10% suspensions in PBS were prepared by sonification as described previously (Bopegamage et al., 2003). Penicillin (50 units ml−1) and 40 μg streptomycin ml−1 were added. Suspensions were incubated overnight at 4°C, centrifuged at 1500 g for 30 min at 4°C, passed through a 0.45 μm Millipore filter and frozen at −80°C. Each homogenate was titrated separately.

**Quantification of infectious virus in organs.** Tenfold dilutions of the sera, organs and stool-pellet suspensions were prepared. Of these diluted suspensions, 100 μl was added (eight wells per dilution) to monolayers of HEp-2 cells grown on 96-well flat-bottom microtitre plates and incubated at 37°C in a CO2 incubator. Virus titres were expressed as TCID50 values, calculated by the method of Reed & Muench (1938). Results were read at days 4–7 after infection. To confirm that CPE was not due to toxicity, each sample was passaged further in HEp-2 cells.

**PCR analysis.** RNA was extracted from the different snap-frozen organs with a GenElute Mammalian total RNA kit (Sigma). cDNA synthesis and cDNA amplification were performed by using a single-tube method with a Sigma Enhanced Avian RT-PCR kit, as described previously (Swanink et al., 1994; Zoll et al., 1992).

**Histology.** Serial 4–7 μm thick sections of formalin-fixed, paraffin-embedded samples of heart, spleen, pancreas and small intestine were stained with haematoxylin and eosin. Cellular infiltration (I) and necrosis (N) in the tissue were graded as described by Opavsky et al. (1999). A score of 0 corresponds to the absence of inflammation or necrosis; 1, incipient, focal inflammation or necrosis (only one or two foci in the whole section); 2, mild to moderate infiltration or...
necrosis (10–40% of section affected); 3, moderate infiltration (40–70% of section affected); 4, extensive areas of infiltration or necrosis (70–100% of the tissue section affected).

Localisation of virus by immunohistochemistry. Staining for VP1 was performed as reported previously (Bopegamage et al., 2003) with some modifications. The specimens were heated three times for 5 min in a 10 mM citrate buffer in a microwave oven. The sections were then cooled and immersed in 3% H2O2 for 15 min to inhibit endogenous peroxidase activity. To block non-specific antigen sites, sections were incubated with Protein Block Serum-Free (DAKO) for 20 min at room temperature. The primary antibody (monoclonal mouse anti-VP1 antibody, clone 5-D8/1; DAKO) diluted 1:250 (after standardization) was mixed with biotinylation reagent (modified biotinylated anti-mouse immunoglobulin) and pre-incubated for 15 min (‘antibody mix’). The Animal Research kit (DAKO) was used to minimize background staining from anti-mouse immunoglobulin. After background blocking, the ‘antibody mix’ was applied to the specimens and incubated for 15 min. Specimens were incubated with streptavidin–peroxidase and again washed with PBS. Diaminobenzidine/hydrogen peroxidase substrate (DAB) was incubated for 2–10 min to reach the appropriate intensity and slides were rinsed with distilled water to stop the staining reaction. Immunostained sections were counterstained and mounted with coverslips as described above. The intensity of staining was scored similarly to that for VP1.

For staining with the anti-3A antiserum, a slightly different method was applied, without using the Animal Research kit. After background blocking, the primary antibody (rabbit antibody against 3A) was diluted 1:100 (after standardization), applied to the tissues and incubated overnight at 4°C. After washing three times in PBS, slides were treated with 6 M urea plus 0·1 M glycine for 30 min and rinsed in PBS. Secondary antibody (goat anti-rabbit immunoglobulin) conjugated to peroxide-labelled dextran polymer (DAKO EnVision) and DAB were used to visualize the reaction. After washing in PBS, slides were counterstained and mounted with coverslips as described above. The intensity of staining was scored similarly to that for VP1.

Localization of IFN-α by immunohistochemistry. The tissue sections were immunostained without using antigen-retrieval methods. The blocking methods were similar to those described above for the VP1 protein. After background blocking, the primary antibody (rabbit polyclonal antibody against mouse IFN-α; PBL) was diluted 1:75 (after previous standardization) and applied to the tissues, and slides were incubated for 30 min. After washing three times in PBS, slides were incubated for 30 min with the secondary antibody (goat anti-rabbit immunoglobulin) conjugated to peroxide-labelled dextran polymer (DAKO). After washing in PBS, slides were counterstained and mounted with coverslips as described above. The intensity of staining was scored similarly to that for VP1 and protein 3A.

Statistical analysis. The 95% confidence intervals were determined for three values (three mice per time point and per organ), which were the means of three repeated titrations at eight wells per dilution; titres were compared by the standard Student’s t-test.

RESULTS

Virus kinetics after oral versus i.p. infection

Irrespective of virus dose or route of infection, all mice became infected, but notable morbidity was not detected. Virus was already detected in serum and in most of the organs at day 3 after inoculation, but generally virus appeared later after oral than after i.p. infection ($P<0·05$; t-test). The shortest period during which virus could be isolated from organs was 14 days and the longest was 49 days (spleen, oral infection; Fig. 1b). As illustrated, virus dose had only a modest effect on virus titre, but titres were higher after i.p. than after oral infection ($P<0·05$; t-test). For most organs (heart, thymus and large intestine are not shown), the difference in titre was only 1·0–1·5 logs, whereas for the small intestine, a difference of 2–3 logs was found.

It is concluded that both infection routes resulted in a systemic infection, but that virus titres were significantly lower after oral infection than after i.p. infection.

Virus detection by PCR

To further investigate a relationship between the route of infection and its duration, individual organs collected on days 56, 63, 98 and 147 p.i. were analysed by nested RT-PCR. The first time point (day 56) was taken 1–3 weeks after cultures became negative. On days 56, 63 and 98, individual mice consistently scored positive for enteroviral RNA. No correlation was found with virus dose (data not shown). The route of infection had little effect on how long viral RNA could be detected in heart, spleen or pancreas. However, a clear effect on duration of infection was observed for the small intestine (Table 1): samples were PCR-positive only after oral infection (12 mice), suggesting that the oral route causes prolonged infection in the small intestine. The numbers of positive mice dropped after 63 days and, after 147 days, all samples invariably tested negative by RT-PCR (24 mice, 120 samples). It is concluded that: (i) a prolonged infection occurs incidentally in various organs (spleen, heart, pancreas) irrespective of the route of infection; (ii) oral delivery causes prolonged infection in the small intestine; and (iii) the infections are eventually cured in all mice.

Histopathological changes in selected organs

In our previous work, mild infiltration and fibrosis were reported in heart tissue during the acute phase of infection, occurring after both oral and i.p. infection (Bopegamage et al., 2003; Vargova et al., 2003). In the present study, these findings were confirmed and virus dose did not influence the outcome significantly (data not shown). In contrast, the route of infection clearly had a differential effect on histopathology in the pancreas, which will be described in more detail.
Pancreas. Upon i.p. infection, mild to severe inflammation and necrosis occurred in the exocrine pancreas, but not in islets (days 3–14 p.i.). The severity of pancreatitis upon i.p. infection was dose-dependent. At the highest dose (5 × 10⁹ TCID₅₀), severe pancreatitis (grade 4; Fig. 2b) was observed, which gradually subsided and was resolved after 14 days. Mice infected at lower doses showed pancreatitis to a lesser degree (Fig. 2c). The pancreases of some mice showed perivascular or periductal inflammation even at later time points (days 28 and 49; Fig. 2d). In some mice, fat replacement was observed at later intervals (days 35, 49 and 56; Fig. 2e). In striking contrast to the pathology observed after i.p. infection, histology remained completely unchanged in pancreas samples of orally infected mice (Fig. 2a).

Small intestine. In contrast to the differences in virus titres and duration of infection, the infection route had little or no influence on histology. After oral infection, Peyer’s patches were enlarged and some inflammation of the intestine was observed, changes that were not found after i.p. infection (data not shown).

Table 1. Enterovirus-specific RT-PCR: results for culture-negative samples

<table>
<thead>
<tr>
<th>Organ</th>
<th>Oral infection (days p.i.)</th>
<th>i.p. infection (days p.i.)</th>
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<tr>
<td></td>
<td>56</td>
<td>63</td>
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<tr>
<td>Heart</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Spleen</td>
<td>1</td>
<td>2</td>
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<tr>
<td>Pancreas</td>
<td>4</td>
<td>0</td>
</tr>
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<td>Small intestine</td>
<td>8</td>
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<td>Large intestine</td>
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Immunohistochemical analysis of pancreas and small intestine for the presence of enteroviral VP1

Although histology revealed no evidence for pancreatitis after oral infection, PCR results for the pancreas were repeatedly positive (Table 1). This finding was further elaborated by systematic investigation of the pancreas and small intestine for the presence of enteroviral antigens (VP1 and protein 3A).

Pancreas. In most, but not all, mice, VP1 was observed in the exocrine as well as endocrine pancreas as early as...
3 days after infection. VP1 was detected irrespective of the route of infection (oral or i.p.) or the presence of tissue damage and inflammation (Fig. 3a–d; yellow to brown colour). The highest intensity (grade 4) for VP1 staining was found at 5–7 days p.i. in all mice, irrespective of pathology. Pancreas tissue of both orally and i.p.-infected mice was still positive for VP1 at day 28, irrespective of virus dose. At day 56 p.i., one-third to one-half of the pancreas samples were still positive (Fig. 3c, d), which is in agreement with the PCR results (Table 1). Staining for protein 3A (a non-structural protein) showed a similar distribution pattern, confirming that VP1 staining was based on active virus replication (Fig. 3e, f). The intensity of 3A staining faded, however, after the acute phase of infection (day 14). Control mice were negative (Fig. 3g, h).

**Small intestine.** VP1 was predominantly observed in the villi (Fig. 3j, k) after both oral and i.p. infection. After i.p. inoculation, the intensity of VP1 staining was lowered by day 14 and scores were generally negative by day 21. After oral infection, the strongest signal was obtained at day 3, regardless of virus dose, and positive signals were frequently observed at later time points (up to day 63 p.i.). The signal prevailed in the smooth muscles of the intestinal wall (Fig. 3l). Positive results were found on day 21 (four of 12 mice), day 28, (four of 12 mice), day 56 (eight of 12 mice) and day 63 (one of 12 mice). Comparable results were found by RT-PCR (Table 1). A characteristic feature of infection after both i.p. and oral inoculation was a total absence of VP1 staining in the Peyer’s patches at all time points (Fig. 3j, k).

**Immunohistochemical analyses of pancreas and small intestine for IFN-α**

Recent publications have pointed to a central role of IFN-α in protection of the endocrine pancreas from damage by viruses (Flodström et al., 2002, 2003). As the pancreas was not affected after oral infection with CVB3, the question of whether expression of IFN-α in the pancreas is associated with and provides an explanation for the observed protection against inflammation and tissue damage was investigated. Indeed, IFN-α expression was seen throughout the observation period in islets as well as exocrine tissue of the pancreases of all mice. The expression of IFN-α was independent of the route of infection, thus offering no explanation for protection. IFN-α was already detected at day 3 and maximum intensity (grade 4) was reached at day 5. The intensity (oral and i.p.) remained unchanged, as illustrated for oral infection at day 56 (Fig. 4c). Control mice were negative for IFN-α (Fig. 4d). IFN-α was also detected in the villi and Peyer’s patches, although the latter were negative for VP1 (data not shown).

**DISCUSSION**

The non-polio enteroviruses can cause overwhelming and potentially fatal disease, with affliction of many organs. A
A similar effect of age has been reported for mice infected by the oral route (Loria et al., 1976). These authors concluded that some kind of intrinsic immunity of the gut matures with age, which provides a barrier to protect the host against overwhelming invasion by the pathogen. Ample evidence currently confirms the existence of a gut barrier, not only in mice, but also in humans, and a role has been reported for the innate immune system combined with a subset of memory effector T cells, which comprise the barrier (Nagler-Anderson, 2001). The early description of the function of a certain gut barrier in coxsackievirus pathology has largely been neglected and the i.p. route of infection became the preferred route to study the pathogenesis of coxsackieviruses in experimental models. Although morbidity may be diminished by oral infection, making this route less convenient for pathogenesis studies, the oral route has much more similarity with the natural (faecal–oral) route of transmission and the mild course of infection observed in humans. Moreover, the existence of a gut barrier may have important consequences for the pathogenesis of coxsackievirus-induced disease. The oral-infection model was therefore revisited and a systematic and explorative comparison was made of both infection routes in Swiss Albino mice for pathology and duration of infection.

Our study shows that a systemic infection can be established readily in Swiss Albino mice by oral delivery and that the route of delivery and the virus dose do not strongly influence the course of infection. A clear effect of infection route, as was expected from the studies of Loria et al. (1976), may have been masked because of low virulence of the CVB3 (Nancy) strain that was used in these experiments. There was no obvious morbidity after i.p. infection and a high severe course is mainly found in neonates and young children, whereas the majority of infections are mild or even asymptomatic, particularly in older subjects (Pallansch & Roos, 2001). Apparently, some kind of immunity is acquired with age, which usually moderates the severity of infection.

Fig. 3. Tissue sections analysed for VP1 and protein 3A. Localization of VP1 and protein 3A was visualized by immunohistochemistry with DAB substrate (yellow–brown colour) and counterstained with haematoxylin and eosin. Pancreas: (a) VP1 staining, day 5, oral route, dose $5 \times 10^7$ TCID$_{50}$; (b) VP1 staining, day 5, i.p. route, $5 \times 10^3$ TCID$_{50}$ (inset, infected islet; bar, 10 $\mu$m); (c) VP1 staining, day 56, oral route, $5 \times 10^9$ TCID$_{50}$; (d) VP1 staining, day 56, i.p. route, dose $5 \times 10^6$ TCID$_{50}$; (e) p3A staining, day 5, oral route, dose $5 \times 10^7$ TCID$_{50}$; (f) p3A staining, day 5, i.p. route, dose $5 \times 10^7$ TCID$_{50}$; (g) VP1 staining, day 3, i.p. route, mock-infected; (h) p3A staining, day 3, i.p. route, mock-infected. Small intestine: (j) VP1 staining, day 7, oral route, dose $5 \times 10^7$ TCID$_{50}$; (k) VP1 staining, day 7, i.p. route, dose $5 \times 10^9$ TCID$_{50}$; (l) VP1 staining, day 63, oral route, dose $5 \times 10^7$ TCID$_{50}$; (m) VP1 staining, day 7, oral route, mock-infected. Bars, 40 $\mu$m (except where indicated).
In contrast to pathology of the pancreas, which was only observed after i.p. infection, inflammation of the small intestine was only observed after oral infection. Peyer’s patches were also enlarged after oral infection. No such changes occurred after i.p. infection, although virus replicated well and even to significantly higher titres (2–3 logs) after i.p. than after oral infection. In spite of lower virus titres, infection of the small intestine lasted much longer after oral delivery: virus could be isolated for 35 days, 2 weeks longer than after i.p. infection (Fig. 1). Moreover, virus remained detectable by PCR and VP1 staining for up to 64 days in the current series and 98 days in another series of experiments (Bopegamage et al., 2003), whereas no evidence was found for prolonged infection of the gut after i.p. infection. Although the oral route of infection correlated with prolonged virus presence in the gut, the latter was not associated with long-term virus excretion in stools, as is known to occur in humans (Chung et al., 2001). Eventually, the mice cleared the infection and they were all negative by 147 days. Thus, significant similarities were observed between the oral route infection of mice and the course of infection in humans, including a prolonged presence of virus in the gut.

As discussed by Harrath et al. (2004), it remains unclear which kind of intestinal cells are the first to replicate virus upon oral delivery. During the acute phase of infection (i.p. and oral infection, day 5), VP1 was mainly located in the epithelial layers of the intestinal mucosa, suggesting that enterocytes are among the first cells in which the virus is replicating (Fig. 3j, k). Previous studies found the virus mainly in stroma of the intestinal villi and proposed that lymphocytes are the first cells to propagate virus (Harrath et al., 2004). However, virus antigens (VP1) were not detected in either stroma or Peyer’s patches, although these stained positive for IFN-α. This observation contrasts with those of Harrath et al. (2004) and also with findings by others who showed that, upon i.p. infection, coxsackievirus preferentially infects B lymphocytes within the gut-associated lymphoid tissue, including Peyer’s patches (Klingel et al., 1996; Mena et al., 1999). It was supposed that B lymphocytes in particular become infected and thereby play a role as Trojan horses in subsequent spreading of the infection. Although our results of immunohistochemistry look clear-cut, double-staining for B-cell markers and VP1 was not performed; thus, it cannot be excluded that some minor population of virus dose affected mainly the pancreas and caused mild inflammation in the heart (Bopegamage et al., 2003). The outcome is consistent with low virulence of the CVB3 strain in Swiss Albino mice. In spite of the overall mild character of the infection, some significant differences were observed between oral and i.p. infection, particularly regarding pathology caused in the pancreas and small intestine.

Intraperitoneal infection caused inflammation and necrosis of the exocrine pancreas, which is consistent with existing literature (Horwitz et al., 1998; Mena et al., 2000; Tracy et al., 2000). Surprisingly, the pancreas remained unaffected when virus was given orally, suggesting indeed that some kind of gut barrier exists that protects the pancreas upon infection via the intestinal route. Virus titres were also higher after i.p. infection, which is largely in agreement with concomitant pathology and bypass of the gut barrier. Unexpectedly, however, these differences were not reflected in the outcome of RT-PCR and staining for viral antigens (VP1 and protein 3A) in pancreatic tissue; virus was detected in damaged as well as unaffected pancreas tissue and, in particular, in the islets, which were invariably protected from damage (after i.p. as well as oral infection). This outcome indicates on the one hand that coxsackievirus, having a well-established cytopathic potential in vitro, is not responsible for necrosis and cell death in vivo, an observation also made by others (Klingel et al., 1996; Mena et al., 2000; Potvin et al., 2003). On the other hand, our experiments showing the presence of VP1 and 3A in islets demonstrate that these cells become infected in spite of a certain level of protection, which holds at least at the microscopic level. Our findings indicate, furthermore, that mouse islet cells are susceptible to CVB infection and that their intactness is not simply due to lack of expression of the coxsackie–adenovirus receptor (CAR) on these cells, as has been proposed previously (Mena et al., 2000). That mouse islet cells are susceptible to CVB infection was also deduced from our previous studies that showed infection of mouse islets in vitro with various CVA and CVB (Bopegamage & Petrovicova, 1994). Moreover, Tracy and co-workers addressed this issue more recently and showed clear expression of mouse CAR in mouse islet cells (Drescher et al., 2004).

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lymphocytes becomes infected, but this requires further investigation.

An intriguing question raised by the outcome of our study is what might be the consequence of a silent infection in mouse islets for the function of β-cells. Does infection of mice with CVB3 (Nancy) impair β-cell function and cause a diabetes-like syndrome, as has been reported for diabeticogenic virus strains (Chatterjee & Nejman, 1988; Horwitz et al., 2003; See & Tilles, 1995; Yap et al., 2003)? Glucose levels were followed up to 98 days after infection with a high dose of CVB3 (Nancy). These measurements revealed normal glucose levels, and thus normal β-cell function, after both oral and i.p. infection (data not shown). A crucial question arising from this study is whether the function of the pancreas would become affected if a diabeticogenic strain of virus is given to the mice by the oral route. Preliminary studies with the CVB4 E-2 strain indicate that infection with a diabeticogenic virus causes a disturbance of glucose homeostasis that is sometimes severe, supporting the idea that virus strain is very crucial indeed (unpublished observations). In conclusion, the oral-infection route mitigates pathology, but does not prevent infection of the pancreas (acinar tissue and islets) and, although silent infection with CVB3 (Nancy) did not impair β-cell function, it may still play a role in the diabetic process by triggering IFN-α and autoimmune in genetically predisposed animals (Devendra & Eisenbarth, 2004). Further studies are required to solve the latter issue. On the other hand, growing evidence points towards unique features of virus strains that render them diabeticogenic, not only in mice, but probably also in humans (Paananen et al., 1979). The nature of these unique features is not yet determined, but investigations are highly warranted, as they may shed light on unexplained issues regarding environmental factors in type 1 diabetes.

Expression of IFN-α in mouse islets has been implicated as the crucial factor in protection of β-cells upon infection with CVB as well as other viruses (Chehadeh et al., 2000; Flostrup et al., 2002, 2003). Therefore, expression of IFN-α in the pancreas upon infection was studied. The localization of virus (VP1 staining and RT-PCR) correlated well with expression of IFN-α in the pancreas. However, expression of virus or IFN-α did not correlate with either pathology (necrosis and inflammation) or protection of exocrine or endocrine tissue. Apparently, expression of IFN-α did not prevent infection, but also did not damage acinar tissue upon i.p. infection. It seems, therefore, that the conditions for protection are more complex and, as well as IFN-α, include additional innate defence mechanisms. Alternatively, the amount of IFN-α or the kinetics of IFN-α expression may be critical for the outcome, factors that have not yet been studied in detail. Chehadeh et al. (2000) showed that expression of IFN-α may result in a persistent CVB infection in human islets, a result that is similar to our in vivo findings in mice. The prolonged infection, which, in our model, is associated with ongoing expression of IFN-α, may provide adverse conditions that promote autoimmunity and diabetes, as has recently been proposed by others (Devendra & Eisenbarth, 2004; Lang et al., 2005).

Another key observation from this study was the prolonged presence of virus in several organs up to day 98 after infection, which was demonstrable by PCR. Moreover, the virus could be localized in tissues by immunohistochemical staining for VP1. The term ‘prolonged’ infection is preferred because mice were invariably negative for virus when tested at later time points (> 98 days), indicating that the infection is cured after some time. Remarkably, a prolonged infection of the small intestine was only observed after oral infection (12 mice after oral infection, but none after i.p. infection). These findings corroborate our preliminary experiments, which showed virus presence by VP1 staining in the muscular layer of the small intestine for as long as 98 days (Bopegamage et al., 2003).

The main conclusions from these explorative experiments are: (i) the oral route of infection moderates pathology in the pancreas and duration of infection in the small intestine, and thereby shows more similarity to infections in humans; and (ii) prolonged virus presence is associated with (prolonged) production of IFN-α, the expression of which does not correlate with tissue protection. It is concluded that the oral route of infection provides a more natural way to explore the role of enteroviruses in pancreatitis and type 1 diabetes mellitus.

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