Tissue tropism of recombinant coxsackieviruses in an adult mouse model

Heli Harvala,1 Hannu Kalimo,2,3 Jeffrey Bergelson,4 Glyn Stanway5 and Timo Hyypia¨1,6

Correspondence
Heli Harvala
h.harvala@vir.gla.ac.uk

1Department of Virology and MediCity Research Laboratory, University of Turku, FIN-20520 Turku, Finland
2Department of Pathology, University of Helsinki and Helsinki University Hospital, FIN-00014 Helsinki, Finland
3Department of Pathology, University of Uppsala, Uppsala, Sweden
4Division of Immunologic and Infectious Diseases, The Children’s Hospital of Philadelphia, 3615 Civic Center Blvd, Philadelphia, PA 19104, USA
5Department of Biological Sciences, University of Essex, CO4 3SQ Colchester, UK
6Department of Medical Microbiology, University of Oulu, PO Box 5000, Fin-90014 Oulu, Finland

Received 11 September 2004
Accepted 11 March 2005

Recombinant viruses, constructed by exchanging the 5’ non-coding region (5’NCR), structural and non-structural protein coding sequences were used to investigate determinants responsible for differences between coxsackievirus A9 (CAV9) and coxsackievirus B3 (CBV3) infections in adult mice and two cell lines. Plaque assay titration of recombinant and parental viruses from different tissues from adult BALB/c mice demonstrated that the structural region of CBV3 determined tropism to the liver tissue due to receptor recognition, and the 5’NCR of CBV3 enhanced viral multiplication in the mouse pancreas. Infection with a chimeric virus, containing the structural region from CBV3 and the rest of the genome from CAV9, and the parental CBV3 strain, caused high levels of viraemia in adult mice. The ability of these viruses to infect the central nervous system suggested that neurotropism is associated with high replication levels and the presence of the CBV3 capsid proteins, which also enhanced formation of neutralizing antibodies. Moreover, the appearance of neutralizing antibodies correlated directly with the clearance of the viruses from the tissues. These results demonstrate potential pathogenicity of intraspecies recombinant coxsackieviruses, and the complexity of the genetic determinants underlying tissue tropism.

INTRODUCTION

Coxsackieviruses, members of the genus Enterovirus of the family Picornaviridae, are amongst the smallest human pathogens, approximately 30 nm in diameter, and consist of an RNA molecule surrounded by a protein capsid. The RNA has a 5’ non-coding region (5’NCR) followed by an open reading frame encoding a polyprotein, which is processed into four structural (VP1–4) and seven non-structural proteins (2A–C, 3A–D; Stanway 1990). After binding to the receptor molecule(s) on the surface of the target cell, the virus is internalised, the genome is released, and translation of viral proteins and multiplication of the viral RNA can begin in the cell cytoplasm. Initial replication of enteroviruses in the body usually occurs in the cells of the respiratory or gastrointestinal tracts. Thereafter, the viruses can reach the bloodstream and cause viraemia, which occasionally leads to infection of secondary target organs such as the central nervous system (CNS), heart, skin, liver and pancreas (Grist et al., 1978).

Coxsackieviruses are divided into two subgroups according to their pathogenesis in newborn mice. Coxsackie A viruses (CAVs) infect skeletal muscle inducing flaccid paralysis, while coxsackie B viruses (CBVs), detected in the CNS, exocrine pancreas, liver, brown fat and skeletal muscle, destroy upper motor neurons, leading to spastic paralysis (Dalldorf & Melnick, 1965; Hyypia¨ et al., 1993). Although all CAV serotypes replicate in suckling mice, and cause myositis in the absence of lesions elsewhere, they have been found to differ genetically from each other (Hyypia¨ et al., 1997; Pulli et al., 1995). The 23 CAV serotypes fall into three genetic clusters: 11 CAVs (e.g. CAV16) constitute Human enterovirus A (HEV-A) species together with enterovirus 71, whereas another 11 serotypes (e.g. CAV21 and 24) belong to the HEV-C species. The remaining serotype,
CAV9, is genetically more closely related to CBVs and echoviruses than to other CAVs, and has been classified as a member of HEV-B species.

Coxackieviruses have been found to be responsible for a wide range of human diseases such as respiratory infections, rashes, myocarditis, myositis, meningitis and encephalitis (Grist et al., 1978), but much less is known about the pathogenesis, tissue distribution and cell tropism of human coxsackieviruses. Interactions between a virus and its specific receptor are determined by viral structural proteins, and are thought to be crucial factors in tissue tropism (Evans & Almond, 1998). Cardiotropism of CBV3 (Cameron-Wilson et al., 1998; Knowlton et al., 1996) and pancreatropism of CBV4 (Caggana et al., 1993; Kang et al., 1994; Ramsingh & Collins, 1995; Yin et al., 2002) have been mapped to the structural proteins, and our previous findings show that the structural proteins of CBV3 determine liver tropism in newborn mice (Harvala et al., 2002). CBVs can make use of at least two cell membrane proteins during their entry: coxsackievirus-adenovirus receptor (CAR; Bergelson et al., 1997), a member of the immunoglobulin superfamily, and decay-accelerating factor of the complement system (DAF or CD55; Bergelson et al., 1995). CAV9 can interact with \( \alpha V \beta 3 \) integrin during cell entry (Roivainen et al., 1994), but it is also able to utilize \( \alpha V \beta 6 \) integrin as a cellular receptor (Williams et al., 2004). In addition to this internalization pathway, dependent on the interaction of the arginine–glycine–aspartic acid (RGD) sequence in the C terminus of the VP1 protein with cell surface integrins, an alternative entry route has also been described (Roivainen et al., 1994; Roivainen et al., 1996). Attenuating mutations in the enterovirus 5′NCR influence translation efficiency, which might be another major factor affecting tropism. For instance, attenuating mutations of polioviruses map to single nucleotides in that region (Minor, 1992), and the virulence determinants of CBV1 in a mouse model (Rinehart et al., 1997) as well as the cardiovascular phenotype of CBV3 (Dunn et al., 2000; Dunn et al., 2003; Tu et al., 1995) have been localized to 5′NCR determinants.

We have previously mapped genetic determinants responsible for the different tissue tropism of CAV9 and CBV3 in newborn mice by using recombinant viruses (Harvala et al., 2002). In the current work we have extended our studies to adult BALB/c mice, and also investigated cell tropism in vitro using cell lines. The results show that the structural proteins of CBV3 are important in the infection of the liver tissue, and the 5′NCR of CBV3 enhances virus replication in the mouse pancreas. We also found that neurotropism correlates with the level of viraemia, which again relates to the presence of CBV3 structural proteins, and these were additionally instrumental in stimulating the formation of high levels of neutralizing antibodies.

**METHODS**

cDNA clones. Molecular cloning and generation of the infectious cDNA of the CAV9 Griggs strain (Chang et al., 1989, Hughes et al., 1995) and the CBV3 Nancy strain (Kandolf & Hofschneider, 1985; Klump et al., 1990), and construction of the recombinant viruses (AAB, ABA, BAA, BAB and BBA) have been described previously (Harvala et al., 2002). These recombinant viruses were constructed by exchanging 5′NCR, structural and non-structural regions between CAV9 and CBV3. As indicated there, for reasons that remain unclear, it was not possible to construct an ABB recombinant. The names of the recombinants indicate the origin of the three distinct regions; for instance, ABA has the 5′NCR from CAV9, the structural protein coding region from CBV3 and the non-structural region and 3′NCR from CBV9.

**Cells.** Green monkey kidney (GMK) cells were used for the preparation of viral stocks and for the determination of virus titres by a plaque assay. The CRL6382 cells originated from the liver tissue of a BALB/c mouse, whereas myoblast cells, C2C12, were derived from the hind limb muscle of a fetal Swiss Webster mouse. C2C12 cells were differentiated to the myoblast and myotube stage of development using growth medium containing 2% horse serum. These cell cultures were used to explore the murine cell tropism of the parental and chimeric viruses. All the cell lines were purchased from the ATCC and maintained as recommended.

**Infectivity titration.** To examine cellular tropism of the chimeric viruses, the mouse cell lines (CRL6382 and C2C12) were infected with 10 p.f.u. of virus stock per cell. The virus titres in samples from the CRL6382 cells, collected after different time intervals, were determined using a CRL6382 cell plaque assay. To investigate growth properties of the viruses in C2C12 cells, in which plaque assay was not successful, monolayers were immunoperoxidase (IP)-stained (Waris et al., 1990) 1 and 3 days after infection using antiserum against heat-treated echovirus 11 (known to react with CAV9 and CBVs; Hyytiä, unpublished).

**Blocking assay.** Antibodies against murine coxsackievirus-adenovirus receptor (mCAR; Bergelson unpublished), decay-accelerating factor (mDAF; International Blood Group Reference Laboratory, Bristol, UK; provided by Seppo Meri, University of Helsinki, Finland) and human integrin \( \alpha V \) subunit (L230, ATCC; obtained from Jyrki Heino, University of Turku, Finland) were used to study the receptor usage of the chimeric viruses in the mouse cell lines. mCAR is a rabbit polyclonal antibody, whereas mDAF and L230 are mouse monoclonal antibodies. CRL6382 and C2C12 cell monolayers were washed once with Hanks’ solution, then antibody dilutions in Hanks’ buffered salts solution (HBSS) containing 0.6% fetal calf serum (FCS) were added, and the cells were incubated at room temperature for 45 min. Approximately 100 p.f.u. of virus stock, diluted in HBSS containing 0.6% FCS and antibody against the mCAR, mDAF or against the integrin \( \alpha V \) subunit was incubated at 37°C for 45 min, added to the CRL6382 cells and incubated at room temperature for 15 min. The solution was replaced with 0.5% carboxymethyl cellulose (CMC; Bionase cellulose gum type 7HFD; obtained from Bröste) in culture medium, and the number of plaques was counted 3 days later. C2C12 monolayers were infected similarly but the proportional number of infected cells was determined by IP-staining.

**Infection of BALB/c mice.** To explore in vivo pathogenicity of the recombinant viruses, groups of 4–6-week-old adolescent male BALB/c mice (groups of 15 mice were infected with each recombinant and parental virus, 120 mice in total), obtained from the Animal Center of the Turku University, were infected intraperitoneally with \( 2 \times 10^5 \) p.f.u. of the chimeric or parental viruses in 100 μl PBS. The groups were maintained separately. The mice were monitored daily and three mice from each group were sacrificed using CO2 euthanasia on days 3, 5, 7 and 12 post-infection (p.i.). Blood and tissue samples from different organs, including pancreas, liver, limb muscle, spleen and spinal cord, were collected aseptically and homogenized in 1 ml PBS. A piece of each tissue was stored at...
Tissue tropism of chimeric viruses

The genomic regions responsible for the differences in tissue tropism and pathogenicity of CAV9 and CBV3 in vivo were investigated through comparison of the outcome of adult mouse challenge with parental (CAV9 and CBV3) and recombinant viruses (AAB, ABA, BAA, BBA and BAB). These recombinant viruses were constructed by exchanging the 5'NCR, capsid and non-structural regions between CAV9 and CBV3 (Harvala et al., 2002). After intraperitoneal inoculation of different viruses, three mice from each group were used to analyse virus multiplication at fixed time intervals in selected organs, including liver, spleen, pancreas, skeletal muscle and spinal cord, and blood, using a plaque assay (Fig. 1, Table 1). Moreover, levels of viraemia in infected animals were compared with the amount of circulating neutralizing antibodies.

**Liver and spleen.** CBV3, ABA and BBA replicated in a highly similar manner in the liver tissue and the spleen (approx. $10^5$ p.f.u. ml$^{-1}$ and 3 days p.i.), whereas no replication was recorded after inoculation of CAV9, AAB, BAA and BAB (Fig. 1, Table 1). This finding showed that the CBV3 capsid is essential for liver and spleen tropism, and suggested that receptor interactions play a key role in the susceptibility of these tissues to infection.

**Pancreas.** As analysed by plaque assay, all parental and chimeric viruses replicated in the mouse pancreas 1 and 3 days p.i. (Figs 1 and 2, Table 1). Virus titres of the CAV9-infected mice remained low (mean titre of three mice 3 days p.i. $6.5 \times 10^4$ p.f.u. mg$^{-1}$) when compared with those observed in CBV3-infected animals ($3.2 \times 10^6$ p.f.u. mg$^{-1}$). The BAA chimera showed increased replication in the pancreas ($4.3 \times 10^6$ p.f.u. mg$^{-1}$) compared with the parental CAV9. The other recombinants exhibited levels of multiplicity in the pancreas similar to that of CAV9, except AAB, which replicated poorly (less than $10^3$ p.f.u. mg$^{-1}$ in the single mouse – in which virus was detected).

**Skeletal muscle.** Multiplication of CBV3, ABA, BAA and BBA strains was detectable in murine thigh muscle 1 and 3 days p.i., whereas no replication was seen in skeletal muscle of animals infected with CAV9, AAB and BAB. The maximum titres ($10^4$ p.f.u. mg$^{-1}$) were measured 3 days after intraperitoneal infection of the mice with the four viruses. Surprisingly, the BAA recombinant replicated in skeletal muscle of only one of three mice at each time point up to 7 days p.i.

**Spinal cord.** While the parental CBV3 and ABA chimera were detected 1 and 3 days p.i. in the CNS by isolation from the spinal cord (approx. $10^4$ p.f.u. mg$^{-1}$), CAV9, AAB, BAA, BAB and BBA strains were not detectable.

**Viraemia and neutralizing antibodies during the course of coxsackievirus infection in BALB/c mice**

CBV3, ABA and BBA were present at high titres in the blood samples at the early stages of the infection ($1.2 \times 10^8$ p.f.u. ml$^{-1}$, $9.9 \times 10^5$ p.f.u. ml$^{-1}$ and $4.5 \times 10^5$ p.f.u. ml$^{-1}$, respectively, Fig. 3). Maximal viral titres in blood were detected 1 day p.i., whereas no signs of viraemia were seen 5 days after the infection. In contrast, BAA and BAB were present at very low concentrations in the blood (less than $10^3$ p.f.u. ml$^{-1}$), while CAV9 and AAB were not detected.

The neutralizing antibody titres against the parental and
Table 1. Tissue tropism of chimeric viruses in adult mice by plaque assay

Each + and – symbol represents the virus titre in the tissues of a single mouse: + indicates that virus was detected in the tissue; – indicates that virus was not detected.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Blood</th>
<th>Pancreas</th>
<th>Liver</th>
<th>Skeletal muscle</th>
<th>Spleen</th>
<th>Spinal cord</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBV3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 day p.i.</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>3</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td></td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>5</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>7</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>CAV9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 day p.i.</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>3</td>
<td>– – –</td>
<td>+ + +</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>5</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>7</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>BAB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 day p.i.</td>
<td>+ – –</td>
<td>+ – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>3</td>
<td>– – –</td>
<td>+ – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>5</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>7</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>ABA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 day p.i.</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ – –</td>
<td>+ + +</td>
<td>+ – –</td>
</tr>
<tr>
<td>3</td>
<td>+ – –</td>
<td>+ + +</td>
<td>+ + +</td>
<td>– – –</td>
<td>+ + +</td>
<td>+ – –</td>
</tr>
<tr>
<td>5</td>
<td>– – –</td>
<td>+ + +</td>
<td>+ + +</td>
<td>– – –</td>
<td>+ + +</td>
<td>+ – –</td>
</tr>
<tr>
<td>7</td>
<td>– – –</td>
<td>+ + +</td>
<td>+ + +</td>
<td>– – –</td>
<td>+ + +</td>
<td>+ – –</td>
</tr>
<tr>
<td>BAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 day p.i.</td>
<td>+ – –</td>
<td>+ – –</td>
<td>– – –</td>
<td>– – –</td>
<td>+ – –</td>
<td>– – –</td>
</tr>
<tr>
<td>3</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>5</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>+ – –</td>
<td>– – –</td>
</tr>
<tr>
<td>7</td>
<td>– – –</td>
<td>+ – –</td>
<td>– – –</td>
<td>– – –</td>
<td>+ + +</td>
<td>– – –</td>
</tr>
<tr>
<td>BBA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 day p.i.</td>
<td>+ – –</td>
<td>+ – –</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>3</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>5</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>+ – –</td>
<td>– – –</td>
</tr>
<tr>
<td>7</td>
<td>– – –</td>
<td>+ – –</td>
<td>– – –</td>
<td>– – –</td>
<td>+ + +</td>
<td>– – –</td>
</tr>
<tr>
<td>AAB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 day p.i.</td>
<td>– – –</td>
<td>+ – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>3</td>
<td>– – –</td>
<td>+ – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>5</td>
<td>– – –</td>
<td>+ – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
<tr>
<td>7</td>
<td>– – –</td>
<td>+ – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
<td>– – –</td>
</tr>
</tbody>
</table>

Fig. 2. Replication of the parental and recombinant viruses in the pancreas of adult mice. The titres of the viruses in different tissue samples were determined using plaque titration. All the viruses studied replicated in the pancreas to titres exceeding $10^3$ p.f.u. mg$^{-1}$, except the AAB recombinant which was able to replicate only to a low titre (less than $10^3$ p.f.u. mg$^{-1}$) in a single mouse.
against the viruses with the CBV3 capsid proteins, which also caused the highest levels of viraemia, was more efficient than antibody response after infection with viruses expressing the CAV9 capsid proteins.

**Growth properties of the recombinant viruses**

The CRL6382 cell line, originating from the liver tissue of a BALB/c mouse, and C2C12 cells, representing skeletal muscle tissue of a fetal Swiss Webster mouse, were used to investigate further cellular tropism of the chimeric viruses in vitro. Growth curves in the CRL6382 cells were determined using a plaque assay (Fig. 4a), while the C2C12 cells were investigated using IP-staining (Fig. 5) because of the lack of virus plaque formation in these cells.

CBV3 and recombinants (ABA and BBA) replicated efficiently in the CRL6382 cells, whereas this cell line did not support the growth of the CAV9, AAB, BAA and BAB viruses (Fig. 4a). To gather information about the interaction between the viruses and their specific receptors in the mouse liver cell line, the ability of antibodies against known CBV receptors to prevent CBV3, BBA and ABA infections in CRL6382 was examined (Fig. 4b). The polyclonal antibody against mCAR blocked the infections efficiently (approx. 70% inhibition), indicating that this cell surface molecule is needed for the infection of the CRL6382 cells. In contrast, antibody against mDAF showed significantly less blocking (<30%). These results are in agreement with the previous findings that an mCAR functions as a receptor for CBVs (Bergelson et al., 1998), whereas human DAF-binding enteroviruses are not capable of interacting with the mouse homologue of DAF (Spiller et al., 2002).

---

**Fig. 3.** Levels of viraemia in mice, infected with parental and recombinant viruses, compared with the titres of neutralizing antibodies. Infectivity titres in blood were determined by plaque assay (right axis) and antibody levels were measured in a microneutralization assay (left axis). The blood samples were collected 1, 3, 5, and 7 days after inoculation of the viruses. ●, Virus titres; bars, antibody titres.
C2C12 cells were differentiated by incubation with 2% horse serum to a stage at which myotube structures became visible. These differentiated C2C12 cells supported the growth of CAV9 and chimeric viruses containing the CAV9 capsid region (AAB, BAA and BAB), while CBV3, as well as the ABA and BBA recombinants, were unable to efficiently infect these cells (Fig. 5). One day after CBV3, ABA and BBA infections, only single cells were seen to be infected by immunostaining; the infection did not proceed, and 3 days p.i. the structure of the myotubes had remained intact. In contrast, the myotubes were destroyed by CAV9, and the AAB, BAA and BAB recombinant viruses 3 days p.i. The antibody L230, dissected against the human integrin αv subunit, blocked the infection of the CAV9, AAB, BAA and BAB viruses in C2C12 cells (data not shown), indicating that αv integrins, which recognize the RGD-motif in their ligands, are needed for the infection of fetal murine skeletal muscle cells.

**DISCUSSION**

Enteroviruses are important and highly prevalent human pathogens, often causing non-specific and mild febrile illnesses, but on occasion they can induce acute infections of the CNS, heart and other organs and, in infants, the course of the disease can be severe due to the multisystem involvement (Grist et al., 1978). Enteroviruses may also have a role in the development of type I diabetes in humans (Hyöty & Taylor, 2002). Studies of the pathogenesis of enterovirus infections in humans are complicated by the fact that many enteroviruses cause similar clinical manifestations, and the same serotype can cause diverse symptoms due to different host and viral determinants. Useful insights into pathogenicity and tissue tropism can be obtained by examining animal models using different virus strains and their genetic modifications. For instance, CAV9 and CBV3 have a different tissue tropism in mice despite their close genetic relatedness (Chang et al., 1989; Harvala et al., 2002; Hyypia et al., 1993). The specific aim of this study was to clarify the molecular determinants affecting the course of coxsackievirus infections in an adult mouse model by using recombinant viruses constructed by exchanging the 5′NCR and structural and non-structural proteins between CAV9 and CBV3.

The interaction between the virus and its specific receptor(s) is determined by the capsid proteins. From our results, the structural genes of CBV3 determine tropism to the liver tissue of adult mice because all recombinant viruses containing the CBV3 capsid replicated efficiently in the liver; in contrast, viruses containing the CAV9 capsid did not, and were not detectable in the mouse liver. A similar pattern of susceptibility was observed when the mouse liver cell line CRL6383 was studied. This finding is in agreement with our previous ISH results, which showed that CBV3, ABA and BBA were detectable in the hepatocytes of newborn mice (Harvala et al., 2002). Several lines of evidence support the hypothesis that the preferential liver tropism of CBVs occurs at the level of virus entry through binding to mCAR, a cell surface protein known to be expressed in mouse liver tissue (Bergelson et al., 1997, 1998); while CAV9 does not recognize CAR. The observation that antibody against mCAR blocked CBV3, ABA and BBA infections in the mouse liver cell line (Fig. 4b), also strongly supports the hypothesis that interaction between the capsid protein of CBV3 and CAR is needed for the infection of hepatocytes.

The pattern of infection of skeletal muscle tissue was more
complex. We found that, although CAV9 and recombinant viruses with the CAV9 capsid proteins can be detected by ISH in skeletal muscle of newborn mice (Harvala et al., 2002), they were generally unable to infect skeletal muscle of adult mice (Fig. 1). The exception was the occasional detection of low levels of the BAA chimera in skeletal muscle tissue until 7 days p.i. The expression patterns of different receptors have been shown to differ during development. The expression of \(aVb_3\) integrin (a receptor for CAV9; Roivainen et al., 1994) has been shown to be downregulated as a part of the myogenic differentiation of skeletal muscle cells in vitro (Blaschuk et al., 1997; Menko & Boettiger, 1987), which could partly explain this difference in CAV9 tissue tropism between newborn and adult mice. However, CBV3, ABA and BBA were able to infect skeletal muscle of both newborn and adult mice, even though mCAR expression is also downregulated during maturation of murine skeletal muscle (Nalbantoglu et al., 1999). Because the CBV3 particles are present at high levels in blood, it may be possible that even a low expression of mCAR is sufficient to facilitate CBV3 entry into the murine muscle cells. Alternatively, CBV3 might be able to utilize another receptor in muscle tissue.

To study muscle tropism further, the mouse muscle cell line C2C12 was induced to a myotube stage of development, which corresponds to the developing stage of muscle tissue of newborn mice. Consistent with the data on CAV infection of newborn mice, these cells supported the infection of CAV9, AAB, BAA and BAB viruses. This cell line is known to express \(aV\) integrins (Kamura et al., 2001) and antibodies against \(aV\) integrins blocked the CAV9 infection of C2C12 cells. Attachment of CAV9 to the \(aVb_3\) integrin is mediated by an RGD tripeptide in the VP1 protein of CAV9 (Roivainen et al., 1991). The RGD motif probably plays an important role in mouse pathogenesis of CAV9, but RGD-independent cell entry routes could also be utilized in the infection of mouse tissues (Harvala et al., 2003; Triantafilou et al., 2000). While RGD-mediated integrin binding is clearly not an absolute requirement for the entry of CAV9 into cells, \(aV\) integrins seem to be an important factor in CAV9 infection of mouse muscle tissue. In contrast, the C2C12 cells were not infected by CBV3, ABA and BBA viruses; this could be related to the known lack of expression of the CAR molecule on these cells (Kimura et al., 2001).

It is well known that CBVs can exhibit high levels of replication in the mouse pancreas and cause acinar cell destruction (Vuurinen et al., 1989), whereas the ability of CAV9 to infect the pancreas has been documented only recently (Harvala et al., 2003). Our results here indicate that CAV9, and all the recombinants except AAB, can infect the pancreas of adult mice (Fig. 2). In agreement with our previous results in newborn mice, the CBV3 5′NCR appears to contribute to pancreotropism in adult mice, since the BAA recombinant showed substantially greater replication in the pancreas of adult mice compared with the parental CAV9 strain. The BAB recombinant also showed approximately 100-fold higher replication in the pancreas compared with the AAB strain. We acknowledge that possible biological incompatibility between the 5′NCR of CAV9 with the non-structural region and 3′NCR of CBV3 may inhibit replication of the AAB chimera in certain specific contexts. However, the replication kinetics of all of the recombinants used in this study (including AAB) were equivalent in standard cell cultures (Harvala et al., 2002), providing evidence that basic replication function is not impaired. Secondly, no specific defects in replication in either human or murine systems were observed between other recombinants that were produced from different 5′ and 3′ ends (BAA and BBA). On the other hand, the replication of the...
parental CAV9 as well as ABA was similar to that of viruses with CBV3 5’NCR, suggesting potentially highly complex interactions between different virus components and the cell. Clearly, intracellular factors are as much involved in tissue tropism as those regions of the virus that mediate entry into the cell. The role of the 5’NCR in pancreotropism could have been more directly demonstrated had the ABB recombinant clone been viable.

Our results may also have implications for human diseases since enteroviruses have been proposed to be involved in the aetiopathogenesis of type I diabetes in humans (Hyötty & Taylor, 2002). In a study on the role of enteroviruses in the onset of type 1 diabetes, antibodies present in the prediabetic period have been reported to be directed against a variety of enterovirus serotypes including CAV9 and CBV3 (Roivainen et al., 1998). Studies of the pathogenic process leading to the development of clinical type 1 diabetes are complicated by differences in cellular tropism of coxsackieviruses for the human and the mouse pancreas. Whereas the endocrine part of the pancreas is thought to be infected in humans (Ylipaasto et al., 2004), coxsackievirus infection in the mouse models is targeted to the exocrine part (Vuorinen et al., 1989). Differences in receptor expression between the mouse and human pancreas may explain these different outcomes of infections. High levels of mCAR expression in murine exocrine pancreas may underlie preferential infection with CBVs (Mena et al., 2000), whereas in humans the infection appears to be dependent on expression of either CAR or αvβ3 integrin in the islets of Langerhans (Ylipaasto et al., 2004). However, in addition to the structural proteins, the CBV3 5’NCR appears to contribute to pancreotropism.

The finding that the 5’NCR has an important role in the pathogenesis of coxsackievirus infections in our mouse model was not surprising, since this genomic region has been shown to contribute to the virulence of other enteroviruses. Attenuating mutations of PVs (nt 480, 481 and 472 in types 1, 2 and 3, respectively; Evans et al., 1985; Gromeier et al., 1999; Minor, 1992; Ren et al., 1991; Svitkin et al., 1990) have been mapped to stem–loop V of the internal ribosomal entry site (IRES), which is responsible for ribosome binding and initiation of protein synthesis in the cap-independent translation. The U→C mutation at nt 234 in the CBV3 5’NCR results in attenuation of cardiovirulence in mice (Tu et al., 1995), while mutation(s) in the CBV3 5’NCR stem–loop II region have been associated with a cardiovirulent phenotype in humans (Dunn et al., 2000, 2003). However, non-cardiovirulent CBV3 chimeras have been found to be pancreovirulent in murine models (Dunn et al., 2003; Tracy et al., 2000). Stem–loop II within the 5’NCR of echovirus 12 was found to be responsible for the growth restriction in primary heart fibroblasts and murine liver cells in vitro, as well as for an attenuated cardiovirulence phenotype in a mouse model (Bradrick et al., 2001; Dunn et al., 2003), whereas the 5’NCR of echovirus 1 conferred a pathogenic phenotype in a transgenic mouse model (Hughes et al., 2003). Comparison of CAV9 and CBV3 5’NCR sequences raises the possibility that the restricted mouse pathogenesis of CAV9 may partly result from differences in the stem–loop II region. Although the predicted structures of stem–loop II are virtually identical in the two viruses, the primary sequence identity is relatively low in this domain (80%), while other stem–loop regions share more than 90% identity and the overall identity of the 5’NCR is 88%.

The occurrence of infectious viruses in the blood is an important stage of virus infection, since it may lead to the infection of secondary target organs. According to our results, the structural proteins of CBV3 were required for the generation of high levels of viraemia in adult mice. Only the parental CBV3 and the chimeric viruses that had a CBV3 capsid caused viraemia in adult mice, and these viruses were also able to infect the mouse spleen. It has been reported that B lymphocytes are an important site of early CBV replication in mice in vivo (Klingel et al., 1996), and CBVs have also been reported to induce a productive infection in B lymphocytes involving approximately 1–10% of the cells (Mena et al., 1999). Although CBV3 replicates to high titres in B- and T-cell lines, the virus does not seem to replicate significantly in peripheral human mononuclear blood cells in vitro (Vuorinen et al., 1994). Therefore, the origin of viraemia during acute enterovirus infections requires further study in animal models and clinical infections.

Enteroviruses may cross the blood–brain barrier and become disseminated to the CNS from the bloodstream, infected leukocytes or neural cells (Feuer et al., 2003). We found that neurotropism correlates with levels of viraemia and the level of virus multiplication (Fig. 6). Parental CBV3 and ABA recombinant were the only viruses able to infect the CNS of adult mice. These viruses were also present at a high level in blood (approx. 10^8 p.f.u. ml^-1), whereas the level of viraemia was approximately 100-fold lower in the BAA, BAB and BBA infections, suggesting that virus concentration in blood may be an important determinant of

![Fig. 6. Comparison of the level of viraemia detected 3 days p.i. with the titres of neutralizing antibodies observed 5 days p.i. Both occurrence of infectious virus in the blood and the neutralizing antibody titres were higher in mice infected with viruses containing the CBV3 capsid genes (CBV3, ABA and BBA) than in CAV9, AAB, BAA and BAB infections.](image-url)
CNS involvement. The latter recombinants exhibited neurotropism in newborn mice (Harvala et al., 2002), which may be due to differences in the development of the blood–brain barrier between adult and newborn mice.

Antibodies seem to be critical in the control of enterovirus infections, and in our work the viraemic phase was terminated by the appearance of maximal neutralizing antibody titres (Fig. 4). It is known that individuals with deficient antibody production are generally able to mount effective immune responses to most viruses, but are susceptible to severe enterovirus infections, such as encephalitis, underlining the role of humoral immunity in protection against enteroviruses (Geller & Condie, 1995; Hertel et al., 1989; Hodes & Espinoza, 1981; Wilfert et al., 1977). The neutralizing antibody titres were much higher in mice infected with CBV3, ABA and BBA than those seen after the CA9, AAB, BAA and BAB infections, indicating that the induction of antibody production by the viruses with the CBV3 capsid proteins is more efficient (Fig. 6). This may be due to the fact that the viruses with CBV3 capsid were present approximately at 100-fold higher level in the blood than the viruses with CA9 capsid proteins, and also caused more severe infections in the mice. These results parallel previous findings in CBV1 infection: the neutralizing antibody response to the virus was consistently higher in mice infected with myopathic strains than with amyopathic viruses (Jongen et al., 1994; Tam et al., 2003). These results suggest that immunogenic determinants may be linked to the pathogenic phenotype of the virus.

In conclusion, the capsid proteins of CBV3 determined tropism to the liver tissue in adult mice, and antibody against the murine homologue of CAR blocked infections by CBV3, ABA and BBA, containing the CBV3 capsid, in the mouse liver cells, indicating that this receptor is a major determinant of tissue tropism. A CBV-like 5′NCR seemed to enhance pancreotropism in mice, although the viruses with the capsid proteins of CA9 were also able to infect the murine pancreas. Neurotropism seemed to correlate with the level of viraemia and with the replication activity of viruses, since only the parental CBV3 and the ABA recombinant replicated to high titres in blood and were able to infect the CNS of adult mice. Our data also clearly demonstrate that neutralizing antibodies are important in the clearance of virus and recovery from coxsackievirus infections in mice.

REFERENCES


