**HepG2 hepatocellular carcinoma cells are a non-permissive system for B19 virus infection**

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Parvovirus B19 has been associated with liver dysfunction and has been considered a potential aetiological agent of fulminant hepatitis and hepatitis-associated aplastic anaemia. The possible effects of B19 virus infection on the liver have been investigated using HepG2 hepatocellular carcinoma cells as a model system, but the reported results are inconsistent. To investigate this relationship further, this study followed the course of B19 virus infection of HepG2 cells in terms of viral DNA, RNA and protein production by quantitative PCR, RT-PCR and immunofluorescence assays. The data showed that B19 virus is able to bind and possibly enter HepG2 cells, but that viral genome replication or transcription is not supported and that viral proteins are not produced. As far as HepG2 cells can be considered a representative model system, any possible pathogenic role of B19 virus on the liver cannot be ascribed to infection or to a direct cytopathic effect on hepatocytes.

Parvovirus B19 is a common human pathogenic virus, associated with a wide range of pathologies and clinical manifestations (Young & Brown, 2004). The virus shows a marked tropism for erythroid progenitor cells in the bone marrow, exerting a cytotoxic effect and causing a block in erythropoiesis that can become apparent as transient or persistent erythroid aplasia in subjects with underlying stressed erythropoiesis or an impaired antiviral immune response. Common manifestations of infection are erythema infectiosum in children or post-infection arthropathies mainly affecting adults; furthermore, the virus has been implicated in a growing spectrum of other pathologies, among them vasculitis, myocarditis, encephalitis and autoimmune connective tissue diseases.

B19 virus infection can be associated with liver dysfunction (Tsuda, 1993) and the virus has been implicated as a causative agent of hepatitis (Hillingso et al., 1998; Naides et al., 1996; Yoto et al., 1996). It has also been investigated as a possible aetiological agent of fulminant hepatitis (Sokal et al., 1998) and hepatitis-associated aplastic anaemia (Langnas et al., 1995; Pardi et al., 1998). However, data on the relationship between B19 virus and liver failure are discordant. B19 virus has been proposed as a possible cause of acute or fulminant hepatitis on the basis of the presence of B19 DNA in liver specimens, but detection of the B19 viral genome by PCR assays in liver tissues cannot be considered a proof of causality. B19 DNA can persist in liver for years following an acute infection and can be detected in a relatively high percentage of individuals (Eis-Hubinger et al., 2001). When liver tissue samples from patients with fulminant hepatitis or hepatitis-associated aplastic anaemia and from control subjects were compared, there was no significant difference in the prevalence of B19 DNA among the groups (Wong et al., 2003). Evidence for B19 virus infection in the course of acute fulminant liver failure has been suggested by the detection in liver tissues of mature virions and viral transcripts in addition to viral genomes (Karetnyi et al., 1999), but other studies investigating the presence of viral transcripts concomitant with viral genomes have reported contrasting results (Abe et al., 2007; Wong et al., 2003), and the presence of viral structural proteins has been documented only episodically (Pinho et al., 2001).

HepG2 hepatocellular carcinoma cells have been proposed as a model experimental system, comparable to primary hepatocytes and therefore suitable for investigation of the effects of B19 virus infection on liver (Caillet-Fauquet et al., 2004; Poole et al., 2004). However, reported results are contrasting. One study (Caillet-Fauquet et al., 2004) reported that infection of HuH7 and HepG2 cells gave rise to a fully productive infection, and hepatocytes have been proposed as a cellular substrate suitable for testing viral infectivity. However, another study (Poole et al., 2004) reported that infection of both primary hepatocytes and HepG2 cells gave rise to an abortive infection, characterized by the absence of viral genome replication, limited expression of the viral genome to the region coding for NS protein and consequent induction of apoptosis. These contradictory results prompted us to investigate...
further the relationship between B19 virus and HepG2 cells as a model system, to test the degree of permissiveness of HepG2 cells and to discriminate the possible pattern of replication and expression of the viral genome. For this purpose, we infected HepG2 cells and followed the course of B19 virus infection in terms of viral DNA, RNA and protein production by quantitative PCR, RT-PCR and immunofluorescence assays.

HepG2 cells (ATCC HB-8065) were cultured in MEM-Earles (Invitrogen) supplemented with 0.1 mM non-essential amino acids (Invitrogen) and 10% fetal bovine serum in a 5% CO₂ atmosphere at 37 °C. The presence of globoside and α5β1 integrins, as possible receptor (Brown et al., 1993) and co-receptor (Weigel-Kelley et al., 2003) for B19 virus, was assessed by indirect immunofluorescence assays. Adherent cells grown on glass slides were fixed in methanol:acetone (1:1) for 10 min and incubated for 1 h with anti-P antigen (GL4 antiserum, IgG/IgM; Matreya), mouse anti-human α5 integrin (MAB-10167; Immunological Sciences) or mouse anti-human β1 integrin (MAB-11019; Immunological Sciences), diluted 1:50 in PBS/0.1% BSA. Cells were washed with PBS and then incubated for 1 h with secondary antibodies (fluorescein isothiocyanate-labelled anti-rabbit or anti-mouse Ig; Dako), diluted 1:20 in PBS/0.1% BSA. The results showed a uniform distribution of receptor and co-receptor molecules on hepatocytes (Fig. 1a–c).

For in vitro infection, adherent cells, grown in disposable multidishes (Nunclon Surface; Nunc) at a density of 10⁶ cells cm⁻², were incubated for 2 h at 37 °C with a reference B19 viraemic serum that did not contain anti-B19-specific IgM or IgG antibodies detectable by ELISA (Biotrin International) in order to obtain a calculated m.o.i. of 10⁻³ viral genome equivalents per cell. Following adsorption, the inoculum virus was removed by washing three times in PBS and the cells were incubated in fresh medium and harvested at selected time points up to 96 h post-infection (p.i.). At each time point, total nucleic acids and an RNA-enriched fraction were prepared and analysed by quantitative real-time PCR and real-time RT-PCR, respectively, for the detection of viral nucleic acids, whilst the production of viral non-structural and capsid proteins was assessed by immunofluorescence assays.

For nucleic acid purification, cell-culture samples corresponding to 500 000 cells were processed either using a MagNA Pure instrument and a MagNA Pure Total Nucleic Acid Isolation kit (Roche), or using a ToTally RNA purification kit (Ambion), following the manufacturers' protocols. As analytical control reagents and reference targets in the amplification reactions, in vitro-synthesized DNA and RNA standards (EC01 sequence) were added to the sample lysis buffer at a concentration of 10⁷ genome equivalents ml⁻¹ (Gallinella et al., 2004). For the analysis of viral DNA, an aliquot of the total nucleic acid fraction, corresponding to 50 000 cells, was amplified directly. For the analysis of viral RNA, an equivalent aliquot of the RNA-enriched fraction was first treated with Turbo DNAfree reagent (Ambion) and then amplified. Real-time PCR and RT-PCR were carried out using a RotorGene

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**Fig. 1.** (a–c) Immunofluorescence assays for the detection of globoside receptor (a), α5 integrin (b) or β1 integrin (c) on the plasma membrane of HepG2 cells. Control cells (insets) were incubated with secondary antibody only. (d–f) Immunofluorescence assays for the detection of NS protein (d), VP1 protein (e) or VP1/2 proteins (f) on HepG2 cells at 72 h p.i. Cells were counterstained with Evans Blue. Original magnification ×40. Bars, 50 μm.
3000 system (Corbett Research) and SYBR Green detection of amplification products. Amplification reactions were performed using a QuantiTect SYBR Green PCR kit (Qiagen) or a QuantiTect SYBR Green RT-PCR kit (Qiagen), including 10 pmol of each specific primer pair (Table 1). For PCR, the thermal profile consisted of 15 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at 50 °C and 30 s at 70 °C coupled with signal acquisition. For RT-PCR, two parallel reactions were performed for each sample, either including (RT+) or omitting (RT−) reverse transcriptase from the reaction mix, and performing an initial step consisting of 30 min at 50 °C before the amplification reaction with a standard thermal profile. A final melting curve was determined, with the thermal profile ramping from 50 to 95 °C at a rate of 12 °C min⁻¹, coupled with continuous signal acquisition. Fluorescence emission was recorded in the FAM/SYBR channel of the instrument and analysed using the functions available in the RotorGene 6.0 software. Melting-curve analysis was used for determination of the specificity of the amplification products by defining, for each reaction, the melting profile and the Tm of the products; specific accumulation of the amplification products was also confirmed by agarose gel electrophoresis analysis. Quantification of viral DNA and RNA was obtained using a calibrated quantification algorithm, normalized to the amount of EC01 (Bonvicini et al., 2006).

Quantification of viral DNA was carried out using the HR2 primer pair. Under our experimental conditions, the amount of virus detected following adsorption (2 h p.i.) was 2 log₁₀ lower than the amount of inoculum virus (0 h p.i.). The amount of viral DNA decreased further at 24 h p.i. and thereafter maintained a constant level for the full time course of infection (Fig. 2a). This observation indicated that HepG2 cells are not permissive for viral replication, in contrast to a previous report (Caillet-Fauquet et al., 2004), but confirming in this respect the results obtained by Poole et al. (2004).

The pattern of transcription was investigated using different primer pairs: two contiguous primer pairs (HR1 and HR2) able to amplify both the viral DNA and RNA and a non-contiguous primer pair (HR6) able to selectively

### Table 1. Primers used in real-time PCR and RT-PCR assays for the detection and quantitative evaluation of parvovirus B19 DNA and RNA

Position and sequence refer to GenBank accession no. NC_000883.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position (nt)</th>
<th>Sequence (5’→3’)</th>
<th>Target</th>
</tr>
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<tbody>
<tr>
<td>HR1 forward</td>
<td>1882–1901</td>
<td>GCGGGAAACTACAACAAACT</td>
<td>DNA and NS RNA</td>
</tr>
<tr>
<td>HR1 reverse</td>
<td>2033–2014</td>
<td>GTCCCAAGCTTTGTGCAATTAC</td>
<td>DNA total RNA</td>
</tr>
<tr>
<td>HR2 forward</td>
<td>2210–2229</td>
<td>CGCTTGAACACTGAAACCC</td>
<td>DNA total RNA</td>
</tr>
<tr>
<td>HR2 reverse</td>
<td>2355–2336</td>
<td>GAAACTGGTCGCGCAAGGT</td>
<td>DNA and total RNA</td>
</tr>
<tr>
<td>HR6 forward</td>
<td>547–566</td>
<td>CCTGGACCTTCTTGCTGTTT</td>
<td>Spliced RNA</td>
</tr>
<tr>
<td>HR6 reverse</td>
<td>2317–2298</td>
<td>CACGATGCAGCCTACAACCTC</td>
<td>DNA and total RNA</td>
</tr>
</tbody>
</table>
amplify the spliced viral RNA. In particular, HR1 detected unspliced viral mRNA, coding for the NS protein; HR2, specific for the common central exon of the B19 virus genome, provided an estimation of the abundance of total viral RNA; HR6 detected all viral RNA that underwent splicing of the leader sequence to the central exon, including mRNAs coding for VP1/2 proteins. For both contiguous primer pairs (HR1 and HR2), the fluorescent signal acquired in RT+ reactions was not significantly different from the corresponding signal in RT reactions for all time points p.i. (Fig. 2b, c); therefore, the amount of viral RNA was constantly negligible compared with the concurrent DNA background. For the non-contiguous primer pair HR6, no amplification products were detected. These results clearly indicated the absence of any significant transcriptional activity of the viral genome, thus contrasting in this respect with the results reported by Poole et al. (2004).

For the immunological detection of viral proteins, HepG2 cells collected at the different time points p.i. were spotted onto glass slides, air dried and fixed with acetone: methanol (1:1) for 10 min at −20 °C. Slides were incubated for 1 h with a monoclonal human antibody against NS protein (a kind gift of S. Modrow, University of Regensburg, Regensburg, Germany), a monoclonal mouse antibody against VP1 protein (MAB829; Chemicon) or a monoclonal mouse antibody against VP1 and VP2 proteins (MAB8293; Chemicon), diluted 1:200 in PBS/1 % BSA. Slides were washed three times in PBS for 3 min each and then incubated for 1 h with fluorescein-conjugated anti-human or anti-mouse secondary antibodies (Dako), diluted 1:20 in PBS/1 % BSA. Under these experimental conditions, normally used for the detection of viral proteins in infected permissive cells (Bonvicini et al., 2006), B19 virus NS and VP1/2 antigens were not detected at any time p.i. (Fig. 1d–f), confirming the absence of expression of the viral genome and the results obtained from viral nucleic acid analysis.

Several studies and case reports have associated B19 virus infection with pathological processes in the liver, mainly because of the detection of B19 DNA in bone marrow, peripheral blood or liver tissue concomitant with liver dysfunction or inflammation (Hillingso et al., 1998; Langnas et al., 1995; Naides et al., 1996; Pardi et al., 1998; Sokal et al., 1998; Tsuda, 1993; Yoto et al., 1996). However, it should be pointed out that B19 DNA can be detected for prolonged periods of time following an acute infection or can be present in a high proportion of tissues from normal control subjects (Eis-Hubinger et al., 2001; Wong et al., 2003). Therefore, to establish whether B19 virus is a mere bystander in liver tissue or whether its presence may be of biological and clinical significance, it is necessary to identify a range of possible target cells and to investigate the characteristics of viral infection in terms of viral genome replication and expression.

Hepatocytes express both the cellular receptor globoside and integrins as co-receptors and can therefore be considered a potential cellular target for B19 virus infection. Our data show that B19 virus is able to bind and possibly enter HepG2 cells. However, under our experimental conditions, the amount of B19 DNA, after an initial decrease, did not increase significantly during the time course of infection, clearly indicating that HepG2 cells cannot support B19 virus replication. This present result differs from previous results (Cailliet-Fauquet et al., 2004), obtained using an end-point nested PCR assay, indicating that HepG2 cells can support viral replication and release viral progeny into the supernatant of infected cells. Our use of a quantitative assay, able to determine any possible variation in the amount of viral nucleic acids over the course of infection, strongly suggests that HepG2 cells should be considered a highly restricted system with respect to viral replication.

The expression pattern of B19 virus in HepG2 cells is another matter of debate. Whilst a productive replicative cycle implicates a full transcriptional pattern of the viral genome, previous studies indicated a restricted pattern of expression in HepG2 cells, characterized by the selective production of transcripts encoding the NS protein, but not of transcripts encoding the VP1/2 proteins (Poole et al., 2004). In our experimental approach, we used a quantitative RT-PCR assay and different contiguous and non-contiguous primer pairs, appropriate to investigate the total amount of viral RNA as well as the presence and relative abundance of selected subsets of transcripts. We could not find any significant level of any viral RNA in infected HepG2 cells, so our results seriously question the possibility that B19 virus has any transcriptional activity in infected HepG2 cells.

Neither NS nor VP viral proteins could be detected in infected cells, so any possible cytopathic effect of B19 virus should not be attributed to differential production of viral NS protein or to its effects on induction of apoptosis (Poole et al., 2006). Other possible mechanisms need to be hypothesized for a possible apoptosis-inducing effect of B19 virus on hepatocytes.

HepG2 hepatocarcinoma cells may not possess the same properties as primary hepatocytes in terms of susceptibility and permissiveness to B19 virus, and therefore in vivo infection of hepatocytes can still be hypothesized as leading to different outcomes. However, from the data obtained in the present study and as far as HepG2 cells can be considered a reliable and representative model system, it is reasonable to infer that any possible causal role of B19 virus in the course of hepatitis cannot be ascribed to infection or to a direct cytopathic effect on hepatocytes.

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References


