Hepatitis E virus (HEV) is a significant cause of acute hepatitis (Purcell & Emerson, 2001). Its 7.5 kb, positive-sense, single-stranded RNA genome is organized as three overlapping open reading frames (Tam et al., 1991). The virus capsid consists of subunits (capsomeres) made up of partial homodimers of a single structural protein of 660 aa, encoded by the second open reading frame (Xing et al., 1999). A truncated peptide, p239 (aa 368–606), of the HEV structural protein was found to occur in neutral solution as 23 nm particles, which dissociate under mildly denaturing conditions into partial homodimers consisting of a dimeric and a monomeric domain (Li et al., 2005a). The latter structure was strongly reactive with acute and convalescent sera from hepatitis E patients. It was shown that some monoclonal antibodies (mAbs) generated against the peptide are reactive against conformational determinants locating to the dimeric domain, whilst others recognize linear epitopes locating to the monomeric domain (Li et al., 2005a). Many of these antibodies were shown to be reactive against HEV, effecting immune capture of the virus, and at least two of them also neutralize infectivity of the virus (Zhang et al., 2005). The peptide was found to protect non-human primates against the virus, and sera from the vaccinated animals neutralized infectivity of the virus (Li et al., 2005b).

In the light of its structural and antigenic resemblance to the HEV capsomeres, we postulated that p239 may serve as a probe for the HEV receptor. We tested this hypothesis by studying binding of p239 to four cell lines that are susceptible to HEV infection and one that is not susceptible. Binding specificity was analysed by using nine mAbs generated against p239 and one mAb generated against each of human immunodeficiency virus (HIV) and hepatitis B virus (HBV). Two putative HEV receptor-binding sites inferred from these findings were analysed further by mutagenesis of the respective sites.

Four of five cell lines used in the present study, namely HepG2 and Huh7 (Emerson et al., 2004), PLC/PRF5 (Meng et al., 1997; Tanaka et al., 2007) and A549 (Huang et al., 1995; Tanaka et al., 2007), were found previously to be susceptible to HEV infection. Another cell line, P815, was tested and found to be not susceptible to HEV (results not shown). As summarized in Table 1, nine of 11 mAbs used here were generated against p239; six of these were found to recognize conformational antigenic determinants associating with the dimeric domain locating to aa 459–606 of p239, and three to recognize linear epitopes, with two of the latter locating to aa 423–443 and the other to aa 403–418 in the monomeric domain (Li et al., 2005a; Zhang et al., 2005; Wu et al., 2007). Seven p239 mAbs were found to bind infective HEV, effecting immune capture of the virus, and at least two mAbs, 8C11 and 8H3, were also found to neutralize infectivity of the virus (Zhang et al., 2005). The other two antibodies were generated against HIV and HBV, respectively, and used as controls in the present study. p239 and two mutant peptides derived from it, Δ239 and p233, were purified for use in this study as described previously (Li et al., 2005a).

Binding of p239 to HepG2 and Huh7 cells was visualized by immunofluorescence using a confocal microscope. Cells cultured on coverslips were reacted with p239

Putative receptor-binding sites of hepatitis E virus

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A truncated structural protein of hepatitis E virus (HEV), p239, occurs as 23 nm particles consisting of partial homodimers. As the latter resemble the HEV capsomere structurally and antigenically, it was postulated that the recombinant protein may serve as a probe for the HEV receptor. This hypothesis was supported by findings that purified p239 bound and penetrated different cell lines that are susceptible to HEV, and inhibited HEV infection of these cells. The binding was blocked by four of six monoclonal antibodies (mAbs) reactive against the dimeric domain of p239, and by two of three mAbs reactive against its monomeric domain, suggesting that binding may involve a portion of each domain. Mutation affecting the monomeric domain had no effect on binding or capacity to block HEV infection, whereas that affecting the dimeric domain diminished binding of the mutant peptide markedly and abrogated its capacity to block HEV infection. These results suggest that HEV infection might involve distinct receptor-binding sites.
Table 1. Blocking of p239 binding to different cell lines by mAbs

p239-specific mAbs recognize conformational determinants (C) locating to the dimeric domain in aa 459–606, or linear epitopes (L) in the indicated locations in the monomeric domain. p239 binding was determined as described in the legend to Fig. 1. +, Capacity of antibodies to effect immune capture of infective HEV particles or to block p239 binding to cells; --, the respective activity was not detectable; NA, not applicable; ND, not done.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Antigenic determinants</th>
<th>HEV immune capture</th>
<th>Blocking of p239 binding to cell line:</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>HepG2</td>
<td>Huh7</td>
</tr>
<tr>
<td>8C11*</td>
<td>p239</td>
<td>C</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8H3*</td>
<td>p239</td>
<td>C</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1A5</td>
<td>p239</td>
<td>C</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8G12</td>
<td>p239</td>
<td>C</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12A10</td>
<td>p239</td>
<td>L (aa 423–438)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>16D7</td>
<td>p239</td>
<td>L (aa 423–443)</td>
<td>+</td>
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<td>p239</td>
<td>C</td>
<td>+</td>
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<td>p239</td>
<td>C</td>
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<tr>
<td>15B2</td>
<td>p239</td>
<td>L (aa 403–418)</td>
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<tr>
<td>4D11</td>
<td>HBV</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2F2</td>
<td>HIV</td>
<td>NA</td>
<td></td>
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</tr>
</tbody>
</table>

*pAntibodies with HEV-neutralizing activity.

(100 µg ml⁻¹) at 4 or 37 °C for different times in the presence or absence of the neutralizing mAb 8C11 at 1 mg ml⁻¹. Cells were washed with PBS, fixed with 4% paraformaldehyde and incubated in 2% goat serum for 15 min at room temperature. Cell-bound p239 was localized by reacting the coverslip cultures at room temperature successively with 10 µg 15B2 ml⁻¹ for 1 h, followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:500; Molecular Probes) for 30 min, and examined under a confocal microscope. Fig. 1(a) shows that p239 was localized to the surface of HepG2 and Huh7 cells when examined after 5 min incubation at 37 °C, that the peptide gained entry to the cytoplasm after 30 min incubation and that the presence of 8C11 abrogated binding. It was further shown that binding also occurred at 4 °C, but the bound peptide did not...
penetrate the cells at the lower temperature (results not shown).

Binding was confirmed by Western blotting. Near-confluent cultures in six-well plates were reacted with purified p239 for 30 min in the presence or absence of p239-specific mAbs or control antibodies at 1 mg ml\(^{-1}\), and washed three times. The cells were lysed in sample buffer containing 1% SDS, boiled, subjected to SDS-PAGE and blotted onto a nitrocellulose membrane. The Western blots were blocked by incubating in 5% skimmed milk for 1 h at room temperature, allowed to react in turn with a p239-specific mAb (15B2) at a 1:2000 dilution for 1 h, followed by alkaline phosphatase-conjugated goat anti-mouse secondary antibody for 30 min. Colour was developed with a mixture of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate. Having been dissociated earlier during sample preparation by heating in the presence of 1% SDS, cell-bound p239 was detected by Western blotting as a 15B2-reactive 27 kDa monomer (Fig. 1b). It was found that p239 bound to all four cell lines that are susceptible to HEV, and binding was blocked by mAbs 8C11 and 8H3. It did not bind to P815 cells, which are not susceptible to HEV.

The specificity of p239 binding to the four susceptible cell lines was analysed by using nine p239-specific mAbs and two control antibodies that were raised against HIV (2F2) and HBV (4D11). Table 1 shows that six p239-specific mAbs blocked binding of p239 to the four HEV-susceptible cell lines, whereas the other three p239-specific antibodies and the two control antibodies did not affect binding of the peptide. The blocking antibodies consist of four that recognize conformational determinants associating with the dimeric domain of p239, and two others that recognize overlapping linear epitopes locating to aa 423–438 in the monomeric domain. This selective blocking by two of three monomer-reactive antibodies and four of six dimer-reactive antibodies suggests that some portion, but not all, of the p239 molecule is engaged in binding and that at least two distinct sites, locating separately to the monomeric and dimeric domains, might be involved in the binding.

Binding of p239 was analysed further by using two mutant peptides (Fig. 2). One of these, p233, was derived from the wild-type peptide p239 by truncation of six C-terminal amino acids. The other, Δ239, was derived by deletion of aa 423–438. Compared with wild-type p239, Fig. 2(b) shows that deletion of aa 423–438 resulted in loss of reactivity with mAb 12A10, which recognizes a linear epitope locating to aa 423–438. Otherwise, Δ239 retains the capacity of the wild-type peptide to form a 52 kDa homodimer (Fig. 2a), as well as reactivity for the other five mAbs associating with the dimeric structure (Fig. 2b). On the other hand, truncation of six C-terminal amino acids from p239 abrogated the capacity of the mutant peptide to form the homodimer. The resulting mutant peptide, p233, occurred as a monomer of 27 kDa (Fig. 2a) and, whilst retaining reactivity for 12A10, it lost reactivity for all of the other five mAbs associating with the dimeric domain (Fig. 2b).

Fig. 2(c) compares binding of wild-type p239 and the mutant peptides to Huh7, HepG2 and PLC/PRF5 cells. Compared with wild-type p239, binding of p233 was much diminished. Moreover, binding of the wild-type peptide was blocked by both 12A10 and 8C11; the mutant peptide was blocked by 12A10 only. In contrast, Δ239 showed a level of binding similar to that for the wild-type peptide; the binding was blocked by 8C11, but not by 12A10.

Wild-type p239 and the mutant peptides p233 and Δ239 were characterized further by their capacity to block HEV infection. The virus used was a genotype 1 strain originally isolated by inoculating a stool specimen of an acute hepatitis E patient intravenously into rhesus monkeys. It was recovered from bile samples of the infected animals, a pooled sample of which was determined to contain 1×10\(^7\) HEV genome copies. Near-confluent cultures of HepG2 and Huh7 cells in six-well plates were infected with 1×10\(^5\) genome equivalents of a strain of genotype 1 HEV (m.o.i. approx. 0.2, namely 1×10\(^5\) genome equivalents of HEV per 5×10\(^5\) cells). In blocking experiments, p239 or its mutant peptide was added to a final concentration of 1 mg ml\(^{-1}\), 30 min before virus inoculation, whilst mAb was mixed with virus inoculum to a final concentration of 1 mg ml\(^{-1}\) and then added to cultures. The infected cultures were incubated for 1 h at 37 °C, washed and incubated further in medium containing 2% fetal calf serum, with daily medium change. Cells were harvested after 6 days and tested by RT-PCR for the presence of the viral sequence as described previously (Zhang et al., 2003). Fig. 2(d) shows that a 190 bp PCR product of the HEV genome was detected in both HepG2 and Huh7 cultures infected with the virus alone, confirming susceptibility of the respective cell lines to HEV, as reported by Emerson et al. (2004). The infection was blocked by the presence of wild-type p239 as well as by the mutant peptide Δ239, whereas p233 did not affect HEV infection.

In conclusion, we have shown that p239 binds to all of four cell lines that have been shown to be susceptible to HEV infection, namely HepG2, Huh7, PLC/PRF5 and A549, but it did not bind to another cell line, P815, that was found to be not susceptible to the virus. Immunofluorescence studies performed with two susceptible cell lines, HepG2 and Huh7, showed that binding occurred initially at the cell surface and that the peptide later gained entry to the cell cytoplasm. It was also shown that cell penetration by the recombinant peptide occurred at 37 °C, but not at the lower temperature of 4 °C. Moreover, the peptide was tested and found to inhibit infection of Huh7 and HepG2 cells. Binding of p239 to four susceptible cell lines was blocked by six of nine mAbs generated against the peptide. These comprised four of six mAbs that are reactive against the dimeric domain of p239, and two of three mAbs that are reactive against the monomeric domain. Thus, binding
apparently involves a portion each of the dimeric and monomeric domains of the p239 partial homodimer. Mutation affecting the monomeric domain had little effect on the capacity of the mutant peptide to bind to cells, and the mutant peptide retained its capacity to block infection by HEV. In contrast, mutation affecting the dimeric domain diminished cell binding markedly and abrogated the capacity of the mutant peptide to inhibit HEV infection. Taken together, these findings support the hypothesis that p239 can serve as a probe for the HEV receptor, and they also suggest that infection may involve distinct receptor-binding sites on the virus capsid.

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