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Analysis of hepatitis E virus neutralization sites using monoclonal antibodies directed against a virus capsid protein

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Abstract

The dimeric form of the recombinant peptide (E2), comprising amino acid 394–606 of the capsid protein of hepatitis E virus (HEV), is strongly recognized by HEV reactive human serum, and when used as a vaccine, it protects rhesus monkeys against experimental HEV infection. In this work, the relationship of E2 to HEV has been probed using three murine monoclonal antibodies, 8C11, 13D8 and 8H3, all of which react predominantly against the E2 dimer, and can effect immune capture of the virus as well. 8C11 and 8H3 were further found to neutralize HEV infectivity in animals. Cross-blocking patterns between these antibodies discerned two spatially separate antigenic domains, one identified by 8C11 and 13D8, and the other, by 8H3. Kinetic studies using BIAcore biosensor suggest that the epitope to which 8H3 is directed is partially masked, and thus has limited access by the native antibody. However, this is not the case with the smaller Fab. Access to the 8H3 epitope was enhanced by the binding of 8C11, and inhibited by the binding of 13D8 to a distal site on the peptide. Similar to the effects of binding 8H3 to E2, 8C11 was found to enhance immune capture by 8H3, while 13D8 was inhibitory. Moreover, 8C11 and 8H3 act synergistically to neutralize HEV infectivity. The parallel cross-reaction patterns that these antibodies exhibit against the peptide and the virus, respectively, implicate two interacting conformationally dependent neutralization sites on the HEV particle. These sites might cooperate in the adsorption and penetration of the HEV virus.

Keywords: Hepatitis E virus; Neutralization sites; Monoclonal antibodies

1. Introduction

Hepatitis E, one of the major causes of acute viral hepatitis in many developing countries, is caused by the hepatitis E virus (HEV), which is transmitted primarily by the fecal–oral route [1]. Anti-HEV antibodies have also been found in a significant number of healthy individuals. Original sporadic hepatitis E cases have also been reported in industrialized countries where HEV is not considered endemic [2–10]. The mortality of acute HEV infection ranges from 0.5 to 1% for the general population to as high as 20% for infected pregnant women [11,12].

Currently unclassified, HEV is a small, non-enveloped virus with icosahedral structure. It contains a positive-sense, single-stranded RNA genome approximately 7.5 kb in length [1]. The viral genome contains three open reading frames (ORFs). Encoding (respectively) a nonstructural protein (ORF1), a 660 amino acid length capsid protein (ORF2) and a small protein with an uncertain function (ORF3) [13]. Phylogenetic analyses have identified four major genotypes [1], but no distinct serotypes have been described to date, and studies

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with monkeys reveal a substantial degree of cross protection between different genotypes [14–17].

Purdy et al. [18] first showed that anti-HEV serum prevented the development of hepatitis. Subsequent studies show that the immunization of monkeys with recombinant ORF2 antigens could confer protection against viral disease [19–21]. This indicates that the capsid protein of HEV contains important neutralization epitopes. Schofield et al. [22] obtained two neutralizing monoclonal antibodies (MAbs) from a phage display library, which was established using experimentally infected chimpanzee marrow, and exhibited HEV neutralization activity. They showed that these antibodies appear to recognize linear epitopes locating to a region between aa 587 and aa 607 of the capsid protein. Meng et al. [23] raised murine antibodies against recombinant ORF2 peptides and showed that the antibodies could neutralize the infectivity of HEV in a cell culture system and, in line with findings of Schofield et al. [22], these authors further showed that the corresponding epitopes were located to a region between aa 452 and aa 617.

E2 was previously characterized as a recombinant peptide comprising aa 394–606 of the HEV capsid protein ORF2 and expressed in *Escherichia coli*. It naturally occurrs as a homodimer and higher oligomer, and is strongly recognized in the oligomeric states by HEV reactive sera from patients [24]. Immunization with the E2 antigen can protect rhesus monkeys against challenges with high doses of HEV, and E2-induced monkey anti-sera can efficiently neutralize the infectivity of HEV in susceptible rhesus monkeys [19,25]. Therefore, neutralization epitope(s) must be present in this recombinant protein.

In the present study, two separate neutralizing conformational epitopes on the ORF2 protein were first identified by three monoclonal antibodies raised against the E2 antigen; each is capable of immunocapture. Interestingly, the binding of one MAb to a neutralization epitope induces changes on the viral capsid that result in the enhanced effectiveness of binding of the other MAb to its neutralizing epitope.

2. Materials and methods

2.1. The production and purification of antigens

The gene encoding pE2 in pGEX vector [24] was subcloned into the pTO-T7 plasmid [26] and expressed as a non-fusion protein in the *E. coli* strain ER2566 (New England Biolabs). The transformant was cultured in LB medium at 25 °C for 5 h and then further incubated for 4 h in the presence of 0.2 mM of Isopropylthio-β-D-galactoside (IPTG). The cells were lysed by sonication. Triton X-100 was added to a final concentration of 2%. The sonicate was allowed to stand at 4 °C for 30 min and centrifuged at 10,000 × *g* for 10 min. The pellet was resuspended in 4 M urea buffer (200 mM Tris, pH 8.5, 5 mM EDTA, 100 mM NaCl, 4 M urea), allowed to stand for 10 min at room temperature and centrifuged at 10,000 × *g* for 10 min. The supernatant was dialyzed overnight against PBS (pH 7.4) and centrifuged at 10,000 × *g* for 10 min. The recombinant protein, termed E2, was purified by high performance liquid chromatogram (HPLC) (System Gold Nouveau, Beckman Coulter Ins.) in a gel filtration column (SW3000, TSK).

Truncated genes were amplified using the following 5′ primers: HEFP (5′ ORF2 aa 394), 5′ ORF2 aa 439 (5′-CATATGGTTATTCAGGATTATGAC-3′), 5′ ORF2 aa 459 (5′-CATATGTCGCGCCCTTTTT-3′) and 5′ ORF2 aa 469 (5′-CATATGGACG TGCTTTGGCTTTCTC-3′). The 3′ primers were HERP (3′ ORF2 aa 606), 3′ ORF2 aa 602 (5′-CTCGAGTTAGGCTAAAACAGCAACC-3′), and 3′ ORF2 aa 600 (5′-CT CGAGTTAAACAGCAACCGCG-3′). The Chinese HEV strain (DDBJ accession number D11092) from which the original E2 gene had been cloned, was used to amplify HEV 0RF2 5′ terminus truncated genes with 5′ aa 459 primer or 5′ aa 469 primer and a common 3′ aa 660 primer (5′-CTCGAGAAATAAACTATAACTCCCGA-3′). The truncated genes were cloned into pTO-T7 and expressed as an E2 antigen.

2.2. The production of MAbs and their Fab fragments

Six to eight-week-old BALB/c mice were given a total of three doses of 5 μg of purified E2 each on days 0, 15 and 30. The first 2 doses were injected i.m. (the first dose was suspended in complete Freund's adjuvant and the second, in incomplete adjuvant). The final dose was injected i.v. without adjuvant and the animals were sacrificed 3 days later. Splenocytes harvested from the animals were fused with sp2/0-Ag 14 mouse myeloma cells in the presence of polyethylene glycol 1500 (Fluka). The cultures were then screened by ELISA using a microplate coated with 30 ng E2 per well, and the bound antibody was detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (DAKO, Denmark). Reactive clones were then subcloned three times by limiting dilution and expanded. The MAbs were produced by injecting 10^6 hybridoma cells into the peritoneal cavity of the BALB/c mice. Ascites fluid was harvested after 7–10 days, centrifuged and purified by treatment with caprylic acid followed by ammonium sulfate precipitation as described [27]. The lmmunoglobulin isotype of the MAbs was determined using an HRP-conjugated isotype specific goat anti-mouse sera (Serotec Co.). The purified MAbs were then labeled with HRP by a periodate coupling method as described [28]. Fab fragments of MAbs were prepared by papain (Sigma) digestion and purified by DEAE column as-described [29].

2.3. Western blotting of MAbs

Purified E2 suspended in a loading buffer (50 mM Tris pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 0.01% bromophenol blue, 8% glycerol) was separated into two tubes. One was incubated in boiling water for 10 min while the other