Putative neutralization epitopes and broad cross-genotype neutralization of *Hepatitis E virus* confirmed by a quantitative cell-culture assay

Suzanne U. Emerson,¹ Pilar Clemente-Casares,¹ Nasser Moiduddin,¹ Vidya A. Arankalle,² Udana Torian¹ and Robert H. Purcell¹

¹Molecular Hepatitis and Hepatitis Viruses Section, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 50 South Drive MSC-8009, Bethesda, MD 20892-8009, USA

²Hepatitis Division, National Institute of Virology, Pune, India

Monolayers of Hep G2/C3A cells were inoculated with genotype 1 *Hepatitis E virus* (HEV) mixed with either anti-HEV or an appropriate control. After 5 or 6 days, cell monolayers were stained with anti-HEV and infected cells were identified by immunofluorescence microscopy and counted. Anti-HEV from vaccinated or infected rhesus monkeys neutralized the virus, as did mAbs that recognized epitopes on the C terminus of a recombinant vaccine protein. Antibodies were broadly cross-reactive, since convalescent serum from animals infected with any one of the four mammalian genotypes all neutralized the genotype 1 virus.

**INTRODUCTION**

*Hepatitis E virus* (HEV) is a non-enveloped, enterically transmitted virus that causes hepatitis E (reviewed by Purcell & Emerson, 2001; Emerson & Purcell, 2003). Molecular characterization of the virus began with isolation of the first cDNA clones (Reyes et al., 1990) and the discovery that the virus contained a positive-sense RNA genome of 7.2 kb. Expression of portions of the genome soon led to the development of various serological assays for anti-HEV (Mast et al., 1998).

Sporadic cases and large water-borne epidemics of hepatitis E occur frequently in many subtropical developing regions of the world, such as India, China and Africa. Epidemics have not occurred in industrialized countries and, until recently, the rare sporadic cases of hepatitis E in these countries were attributed to importation following a visit to an endemic country. However, seroprevalence studies have indicated that anti-HEV is present in 1–20% of the population in industrialized countries (Purcell & Emerson, 2005). Recent studies have demonstrated that HEV is endemic in swine worldwide and suggested that hepatitis E is a zoonosis (Meng, 2000). HEV has been transmitted to humans via ingestion of undercooked sika deer or wild boar meat (Tei et al., 2003; Takahashi et al., 2004).

Hepatitis E is a self-limiting, acute disease that is clinically indistinguishable from that caused by hepatitis A virus. Overall, mortality is less than 1%, with the exception of cases in pregnant women, which can reach a mortality of 20% (Purcell & Emerson, 2001). Because hepatitis E is a significant economic and health burden in countries in which the virus is endemic and because the threat of a potential zoonosis has not been fully evaluated, there is a need for further characterization of the virus and the disease it causes.

Four genotypes of HEV that infect humans have been identified (Emerson et al., 2004). Two of these, genotypes 3 and 4, have also been isolated from swine. The four genotypes apparently comprise a single serotype. However, this has been difficult to confirm because a cell-culture system that could be widely used to quantify virus neutralization is lacking.

In *vitro* neutralization tests were developed that used RT-PCR to detect virus, but these tests had shortcomings (Tam et al., 1997; Meng et al., 1998). One test was not available to most laboratories because it required primary hepatocytes isolated from cynomolgus monkeys and cultured under fastidious conditions (Tam et al., 1997). Another test used a human hepatocarcinoma cell line (PLC/PRF/5) and assayed virus binding by a two-step PCR (Meng et al., 1998). Although these cells are readily available, the PCR part of the assay is, at best, semi-quantitative. Additionally, only those antibodies that block binding of virus to cells would be scored as neutralizing; antibodies that neutralize at a step after binding would not be seen as neutralizing in this test. Also, this test does not discriminate between virus merely adsorbed to cells and that bound to a functional receptor that leads to productive infection.

We have developed a neutralization assay for HEV that uses a continuous cell line (HepG2/C3A) that is readily available and easy to culture. Only replicating virus is detected, thus
ensuring that a biologically relevant receptor is used and that neutralization post-attachment can be detected. The assay was used to characterize neutralizing antibodies to HEV further.

METHODS

Rhesus monkey neutralization test. Production of anti-HEV FAbs and the neutralization assay were described previously (Schofield et al., 2000). Both the antigen used for immunization of a chimpanzee and the panning antigen used to select FAbs from the corresponding phage-display library were from the Sar55 strain (genotype 1). Two antigenic sites were defined previously by indirect competition ELISA with the Sar55 recombinant ORF2 protein (Schofield et al., 2003). For the neutralization test, 400 µl 10% BSA in PBS (10% BSA/PBS) containing 64 50% monkey infectious doses (MID50) of strain Sar55, Mex14 or Meng HEV and 200 µg purified FAb were incubated for 1 h at room temperature and then overnight at 4 °C. Six hundred microlitres PBS was added to each mixture just before intravenous inoculation into an HEV-seronegative rhesus monkey. Seroconversion to HEV, as determined by ELISA to capsid protein (Engle et al., 2002), was used as the criterion for infection. Monkeys were housed at Bioqual (Rockville, MD, USA). The housing, maintenance and care of the animals met or exceeded all requirements for primate husbandry as specified in the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996).

Virus. All virus pools were made as 10% stool suspensions in 10% monkey or calf serum in PBS. All pools had been previously titrated to an approximate 10-1 titre. Virus. All virus pools were made as 10% stool suspensions in 10% monkey or calf serum in PBS. All pools had been previously titrated to an approximate 10-1 titre. Sar55 pools (both genotype 1) each had an MID50 of 105-5 per 0.5 ml of 10% suspension. Virus pools to be inoculated onto cell cultures were clarified by centrifugation for 2 min at 16 000 × g to remove bacteria.

Sera. Serum was collected from four pairs of naive rhesus monkeys that had been experimentally infected with HEV genotype 1, 2, 3 or 4 virus (Zhou et al., 2004) and from two rhesus monkeys that had been vaccinated with an alum-adjuvanted vaccine (Zhang et al., 2004) and from two rhesus monkeys that had been vaccinated with an alum-adjuvanted vaccine (Zhang et al., 2004) and from two rhesus monkeys that had been vaccinated with an alum-adjuvanted vaccine (Zhang et al., 2004) and from two rhesus monkeys that had been vaccinated with an alum-adjuvanted vaccine (Zhang et al., 2004). Serum was collected from four pairs of naive rhesus monkeys that had been experimentally infected with HEV genotype 1, 2, 3 or 4 virus (Zhou et al., 2004) and from two rhesus monkeys that had been vaccinated with an alum-adjuvanted vaccine (Zhang et al., 2004) and from two rhesus monkeys that had been vaccinated with an alum-adjuvanted vaccine (Zhang et al., 2004) and from two rhesus monkeys that had been vaccinated with an alum-adjuvanted vaccine (Zhang et al., 2004). Sera were selected for testing based on a high titre in ELISA.

Radioimmunoprecipitation assay (RIPA). Plasmids encoding methionine followed by amino acids 449–607 of the Sar55 capsid protein (or the corresponding amino acids of Mex14; GenBank accession no. M74506) followed by a translation stop codon were generated by PCR from Sar55 plasmid pSK-HEV-2 (GenBank accession no. AF444002) or by RT-PCR of a cDNA sequence containing Mex14 virions. Four chimeric genomes were produced by fusion PCR using plasmid pSK-HEV-2 as a template.

Plasmids were cloned into the pGEM-T Vector System II (Promega), purified and then verified by sequencing. Plasmids were transcribed and translated with the TNT Coupled Reticulocyte Lysate System (Promega) with SP6 polymerase and [35S]methionine according to the manufacturer’s directions. RIPA were performed as described previously (Schofield et al., 2003). 35S-Radiolabelled peptides were incubated with purified anti-HEV FAB in denaturing RIPA buffer and precipitated peptides were electrophoresed on 12% PAGE Novex gels and detected by autoradiography.

Cell-culture neutralization test. Infection of these cells was described previously (Emerson et al., 2005). HepG2/C3A cells (CRL-10741) obtained from the American Type Culture Collection were grown in MEM with Hanks’ salts, 1-5 g sodium bicarbonate l−1, 0.1 mmol non-essential amino acids l−1, 1 mmol sodium pyruvate l−1 and 10% heat-inactivated ultralow-IgG calf serum (Invitrogen), in a CO2 incubator at 37 °C. Confluent cells were trypsinized and diluted 1:8 in medium and 0.1 ml was carefully added to 0.4 ml medium already in a well of a Nunc LabTek 11 CC2 eight-well chamber slide (Nalge Nunc International). Slides were incubated overnight at 37 °C to provide a sparse population of attached cells. Meanwhile, duplicate samples containing 5 µl serum (undiluted or diluted with PBS) or 20 µl purified FAB in PBS were mixed with 50 µl virus in 10% BSA/PBS and incubated at 4 °C overnight. Next, 50 or 30 µl, respectively, of cell-culture medium was added to the rhesus FAB sample and the tubes were randomly coded by a technician not otherwise involved in the study. The medium was aspirated from the wells of a chamber slide and replaced with samples. Slides were incubated at 34.5 °C for 1 h and the supernatant was then aspirated and replaced with 0.4 ml culture medium supplemented with 2% DMSO, 100 U penicillin ml−1, 0.1 mg streptomycin ml−1 and 0.1 mg gentamicin ml−1. Cells were cultured at 34.5 °C in 5% CO2 for 5–6 days. Slides were rinsed with PBS and cells and were fixed by immersing the slides in acetone at room temperature. Conventional-phase slide collected from a chimpanzee infected with HEV (anti-ORF2) or hyperimmune rabbit serum was raised against the C-terminal 33 amino acids of ORF3 (anti-ORF3) was diluted with 5% BSA in PBS and incubated with air-dried, fixed cells at room temperature for 20 min. Slides were rinsed with PBS and flooded with a solution of Alexa Fluor 488-conjugated goat anti-human IgG or Alexa Fluor 568-conjugated goat anti-rabbit IgG (Molecular Probes). After 20 min at room temperature, the cells were washed with PBS and covered with Vectashield (Vector Laboratories) (Fig. 1). Cells stained with Alexa Fluor 488 were counted using a Zeiss fluorescent photomicroscope and a fluorescein isothiocyanate filter set by manually scanning the entire well at 25× magnification and tabulating the number of fluorescent cells. Pairs of stained cells that appeared to have just divided were counted as one cell. The code was broken only after all samples were counted.

RESULTS

Limitations of an in vivo neutralization assay

Previously we identified two mAbs to the capsid of HEV strain Sar55 (genotype 1) as neutralizing and two others as non-neutralizing based on their ability to prevent homologous infection of a rhesus monkey (Schofield et al., 2000, 2003). In those studies, a mixture of purified FAB and the Sar55 strain of HEV was incubated overnight and injected intravenously into a monkey. Since the two antibodies that neutralized the virus had been mapped to a common antigenic site, which was distinct from that recognized by the two antibodies that did not neutralize the virus, these results appeared reasonable (Schofield et al., 2003). However, attempts to repeat this neutralization test with two other FAbs that were predicted to neutralize HEV gave inconsistent results.

One of the original neutralizing FABS (HEV31) and two putative ones (EBL16 and EBL56) were tested for their ability to prevent infection of rhesus monkeys when 200 µg of each was mixed with 64 MID50 of a genotype 1, genotype 2 or genotype 3 virus to produce nine different combinations. Of the nine animals inoculated with a mixture of virus and FAB, only two were protected from infection as indicated by failure to seroconvert to anti-HEV (Table 1). In both cases,
the protective antibody was HEV31, the one previously found to neutralize a genotype 1 virus; in the current test, it again neutralized the genotype 1 virus and also neutralized the genotype 2 virus. However, it did not neutralize genotype 3 virus even though the affinity of FAb HEV31 for the genotype 1 and 3 recombinant capsid proteins was virtually identical (Kd of 0.8 and 1.3 nM, respectively) (Schofield et al., 2003). The failure to neutralize the genotype 3 strain suggested that there were limitations to the in vivo assay and rendered the other negative results suspect.

A major drawback of the in vivo test (besides its cost and duration) is that it requires that every infectious virus particle be neutralized. The test cannot discriminate between almost total neutralization and the absence of neutralization. Therefore, any test that permitted quantification of residual infectious particles would present a significant advantage.

**Tissue-culture-based neutralization assay**

Although the process is inefficient, cultured HepG2/C3A cells can be infected with HEV (Emerson et al., 2005), so we used these cells to develop a neutralization test for anti-HEV. A time-course was performed to determine the best time to examine the cells. Two wells each of cells on four separate slides were infected and cells on one slide each were fixed on different days. Fixed slides were stored at -20 °C and all slides were then prepared for immunofluorescence microscopy at the same time. Both ORF2 and ORF3 proteins were stained in the majority of cells (Fig. 1), but cells were counted based on ORF2 protein staining only and duplicates were averaged. The mean numbers of ORF2 protein-stained cells were 10, 63, 195 and 185 at 24 h, 48 h, 4 days and 6 days post-infection, respectively. Since the number of stained cells increased until day 4 and then reached a plateau, cells were subsequently fixed on day 5 or 6 post-infection to ensure that the maximum number of infected cells was identified.

Calculations based on RT-PCR-derived genome titres, infectivity titres for rhesus monkeys and the number of HepG2/C3A cells infected suggested that one tissue culture infectious dose was equivalent to approximately 100 MID50 or 10 000 virus genomes. Because infection is so inefficient, only high-titre stocks of virus and of antibody could be tested. A stock preparation of the Akluj strain of a genotype 1 virus isolated in India was used for most experiments because it had the highest infectivity titre and greatest volume of any of our virus stocks. Preliminary studies demonstrated that the HepG2/C3A cells could be consistently infected with this virus but that the number of cells infected could vary by as much as 20-fold from experiment to experiment. We were unable to control this

<table>
<thead>
<tr>
<th>FAb</th>
<th>Rhesus assay</th>
<th>Tissue-culture assay</th>
<th>RIPA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sar55</td>
<td>Mex14</td>
<td>Meng</td>
</tr>
<tr>
<td>HEV4</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>HEV31</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>EBL16</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EBL56</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EBL2</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>EBL89</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*aReported previously by: a, Schofield et al. (2000); b, Schofield et al. (2003).*

**Table 1. Reactivity of selected FAbs**

The FAbs listed are chimpanzee FAbs to the capsid protein of HEV strain Sar55. The rhesus assay measures virus neutralization by inoculating rhesus monkeys with a mixture of virus and FAb and scoring for infection by seroconversion to anti-HEV. The tissue-culture assay measures virus neutralization by plating the virus/FAb mixture on HepG2/C3A cells and counting the number of infected cells. The RIPA measures precipitation of neutralization peptide (amino acids 458–607 of Sar55 or Mex14 virus) translated in vitro. Tested virus strains are Sar55 (genotype 1), Mex14 (genotype 2), Meng (genotype 3) and Akluj (genotype 1). +, FAb neutralized virus or precipitated peptide; -, FAb did not neutralize virus or did not precipitate peptide. ND, Not done.

**Fig. 1.** Examples of HepG2/C3A cells at 6 days post-infection, doubly stained for capsid protein (green) and ORF3 protein (red). The same cell is shown in the upper and lower panel of each pair and all cells are surrounded by a monolayer of non-infected cells.
variability, which most likely was due to subtle differences among cells from different passages.

In order to determine the amount of antibody that would be required to produce a convincing decrease in the number of infected cells, we tested 10-fold dilutions of sera from two rhesus monkeys that had been vaccinated with a recombinant capsid protein of the genotype 1 strain Sar55. The reciprocal ELISA titres of the pre-vaccination and post-vaccination sera for each monkey were <100 (limit of sensitivity) and 10,000, respectively. Although the maximum number of infected cells per well in this experiment was only 34, there was convincing neutralization by the two post-vaccination sera tested at a final dilution of 1:10 and by one of the sera tested at 1:100, but by neither of the sera tested at 1:1000 (Fig. 2).

These results suggested that demonstration of neutralization capability against this stock of virus would most likely require antibody preparations with a reciprocal ELISA titre of 10,000 or greater. They also provided a direct confirmation that the recombinant capsid protein vaccine induced neutralizing antibodies.

**Cross-genotype neutralization**

Vaccination with genotype 1 capsid protein protects rhesus monkeys from challenge by virus of any one of the three mammalian genotypes tested (Purcell et al., 2003). Therefore, antibodies induced following infection with virus of any one of the four genotypes might be expected to neutralize a genotype 1 virus. Since we had convalescent sera from rhesus monkeys infected with each of the genotypes, we could test this prediction in the cell-culture neutralization assay.

Convalescent serum from pairs of rhesus monkeys infected with genotype 1, 2, 3 or 4 virus was tested in a single experiment. All convalescent sera had a reciprocal ELISA titre of 10,000 except that from H389, which was 10-fold lower. In each case, the paired control serum was collected from the monkey 1 week before or on the day of virus inoculation. The cell-culture neutralization assays demonstrated that, compared with their paired pre-inoculation control serum, all convalescent sera were able to neutralize the genotype 1 HEV by 75–95%, except that of H597, which neutralized 50% of the virus (Fig. 3).

In a separate experiment, the same pairs of sera from H402 and H389 were retested along with additional paired sera. Although the number of cells infected this time was 20 times greater than previously, the mean percentage of virus neutralized was comparable in the two assays (data not shown): H402 serum neutralized 88% of the virus in both experiments and H389 serum neutralized 67% of the virus in the second test compared with 75% in the first test. Finally, a second serum from H400 that had a titre of 1000 (10-fold lower than the first H400 serum tested) neutralized only 2% of the virus, compared with 78% neutralization with the higher-titre serum.

These results indicated that broadly cross-reactive neutralizing antibodies are produced by infection with HEV. They also reinforced the conclusion that a minimum reciprocal ELISA titre of 10,000 was optimal, although not always

---

**Fig. 2.** In vitro neutralization of strain Akluj by serum from monkeys vaccinated with strain Sar55 capsid vaccine. Bars indicate the mean number of positive cells in duplicate samples; asterisks indicate individual results. Open bars represent control pre-vaccination sera and shaded bars represent post-vaccination sera. The reciprocal of the final dilution of serum is given below.

**Fig. 3.** Cross-genotype neutralization of genotype 1 strain Akluj by convalescent sera from pairs of monkeys infected with each of the four genotypes. The identification number of the infected rhesus and the genotype of infecting virus are shown below. See Fig. 2 legend for further details.
required, for demonstrating neutralization in this assay. Equally important, they showed that the experiment-to-experiment variability in the number of cells infected did not appreciably affect the outcome.

**Characterization of FAbs to HEV**

This neutralization test provided us with the opportunity to revisit the question of which of the 13 mAbs from our combinatorial library described previously (Schofield et al., 2000, 2003) could neutralize HEV. Based on the data from the four FAbs used in the rhesus test, we had predicted that the seven FAbs that were mapped to antigenic site 1 would not neutralize HEV, that the five FAbs mapped to site 2 would neutralize and that EBL1, which mapped to an epitope overlapping both sites, might neutralize (Schofield et al., 2003). The histidine-tagged FAbs were purified as before on nickel-agarose columns and concentrations were adjusted so that each preparation contained a similar amount of antibody as ascertained by visual inspection of a Western blot of the antibodies developed with an anti-histidine antibody. Nine samples of sufficient volume were also quantified by the BCA protein assay and shown to differ.

A neutralization assay testing all of the antibodies in the same experiment was performed under code. Those FAbs predicted to neutralize (HEV4, HEV31, EBL1, EBL9, EBL16 and EBL56) were diluted 5-fold compared with those predicted not to neutralize. Even though they contained a higher concentration of antibody, samples containing the FAbs that mapped to antigenic site 1 did not neutralize the virus, as had been predicted (Fig. 4). EBL1 also did not neutralize the virus in this test or in a subsequent test when it was diluted only 2-fold instead of 5-fold. In contrast, four of the five FAbs that had been mapped to antigenic site 2 effectively neutralized the virus. Surprisingly, HEV31 was the only antibody in this group that did not neutralize. This result was totally unexpected, since HEV31 was able to neutralize both the Sar55 and Mex14 strains completely in the rhesus test.

The HEV31 plasmid sequence was confirmed and repeat tests using two new preparations of HEV31 were performed. They also failed to demonstrate neutralization by HEV31, whereas HEV4, which had also neutralized Sar55 in the rhesus test, consistently neutralized over 90% of the virus in the cell-culture test, even at a 1: 20 dilution (data not shown).

**Sar55 is not neutralized by HEV31 in the cell-culture assay**

The virus used in the rhesus neutralization assay was the Sar55 strain (GenBank accession no. AF444002), whereas that used in the cell-culture test was the Akluj strain (GenBank accession no. AF124407). Although both viruses are genotype 1, they differ at two positions within the 148 aa ‘neutralization peptide’; this peptide is the shortest capsid peptide of Sar55 that displays the conformational epitopes recognized by HEV31 and HEV4 (Zhou et al., 2004). Since the two FAbs recognize different epitopes within the same region, it was possible that the Akluj strain was missing the epitope recognized by HEV31 but did have the one recognized by HEV4. The hypothesis that the different results with HEV31 in the two tests reflected the virus strain used was tested by comparing the ability of the two FAbs to neutralize Sar55 infectivity for HepG2/C3A cells.

The new neutralization test was performed as described previously except that the strain of virus used was Sar55 instead of Akluj. HEV4 neutralized 99% of this virus (Fig. 5). In contrast, HEV31, tested at two dilutions, decreased the number of Sar55-infected cells by only 38 and 28%, respectively. Although this was more than the 13% observed when HEV31 was tested against the Akluj strain (Fig. 3), it was minimal given the normal variation in the assay and suggested that something other than strain differences accounted for the ability of HEV31 to neutralize virus so effectively in the rhesus assay and so inefficiently, if at all, in the cell-culture assay.

**Attempt to identify amino acids critical for conformational neutralization epitopes**

Since HEV31 but not EBL16 or EBL56 had neutralized the Mex14 strain in the rhesus test, it would be informative to determine their reactivity against Mex14 in the cell-culture assay. Unfortunately, we have not yet been able to generate a stock of Mex14 with a high enough infectious titre to use in
this test. As an alternative, we attempted to exploit naturally occurring amino acid differences to determine which epitopes were common to the Sar55 and Mex14 strains.

There are 12 amino acid differences between Sar55 and Mex14 within the 'neutralization peptide' spanning amino acids 458–607. Since this is the shortest peptide recognized by HEV4, HEV31, EBL16 and EBL56, selected amino acids unique to the Mex14 strain were introduced into the Sar55 peptide by PCR mutagenesis and four chimeric peptides and the corresponding authentic Mex14 and Sar55 peptides were tested by a RIPA with the four FAbs. The four chimeric peptides were all precipitated by antibodies HEV4, HEV31, EBL16 and EBL56 but not by EBL5, a control FAb that maps to antigenic site 1. Chimeric peptides in (a) are designated by position(s) of the amino acid preceded by the single letter code for the Sar55 amino acid and followed by the code for the Mex14 amino acid to which it was mutated: C1 (V499I); C2 (T517S); C3 (S527P, I592V and Q530E); C4 (H604R).

**DISCUSSION**

The cell-culture-based neutralization test for HEV is valuable for providing quantitative data in a relatively rapid and economical manner. We have used the test to confirm directly conclusions that had been drawn earlier based on indirect evidence.

We first confirmed that vaccination with our recombinant capsid protein did indeed induce neutralizing antibodies. Prior to this study, this conclusion was based on data from pre-clinical studies that showed a good correlation between the ELISA titre to HEV capsid protein and protection of rhesus monkeys from challenge with HEV (Purcell et al., 2003). Titration of the immune sera demonstrated that a reciprocal ELISA titre of anti-capsid antibody of approximately 10 000 was required to neutralize most of the input virus in the cell-culture assay. This result was not unexpected, since our calculations indicated that only one of approximately 10 000 virions was able to infect the HepG2/C3A cells and that the abundance of non-infecting virions would sequester much of the antibody.

Additional pre-clinical trials in rhesus monkeys had produced evidence that at least three of the four mammalian genotypes of HEV represented a single serotype. In that study, vaccination with a genotype 1 capsid peptide had protected monkeys from challenge not only with a genotype 1 virus, but also with a genotype 2 or genotype 3 virus (Purcell et al., 2003) (genotype 4 was not available for testing at that time). The current studies complemented those studies and extended them to genotype 4. In this reciprocal test, infection with any one of the four mammalian genotypes induced antibodies that cross-neutralized a genotype 1 virus in the cell-culture assay. Therefore, it appears that all four genotypes of HEV share at least one immunodominant neutralization epitope (Zhou et al., 2005).

Development of the cell-culture neutralization assay also permitted us to evaluate further our panel of anti-HEV mAbs. The panel of anti-HEV recombinant FAb5s generated in chimpanzees has been very important for analysing HEV antigenicity (Schofield et al., 2003). Initially, the panel was
used to define two non-overlapping antigenic sites (site 1 and site 2) on the capsid protein. Limited neutralization tests in rhesus monkeys led to the prediction that antibodies would not or would neutralize HEV depending on whether they recognized site 1 or site 2 epitopes, respectively. As a further extension, a surrogate ELISA for neutralizing antibodies was developed utilizing the smallest peptide which reacted with the five putative neutralizing antibodies (Zhou et al., 2004). The present study has validated this surrogate test, since it confirmed that convalescent sera that reacted with the ‘neutralization peptide’ did indeed neutralize genotype 1 HEV in the cell-culture test. Furthermore, all five FAbs that reacted with this ‘neutralization peptide’ neutralized genotype 1 HEV in the cell-culture test and/or in the rhesus test. The ability of these FAbs to precipitate both the genotype 1 and 2 peptides in RIPA suggests that these FAbs most likely would also neutralize a genotype 2 virus. Therefore, it appears that there is a dominant and perhaps a single antigenic site for neutralizing antibodies and that they can be easily identified and quantified by the ‘neutralization peptide’ ELISA.

In the majority of cases, even when neutralization was efficient, a few cells in culture were still infected. Thus, it is not too surprising that FAbs ELB56 and EBL16 did not appear to neutralize in the rhesus test, since even a few infectious viruses would initiate an infection and spread to new cells in vivo and any neutralization would be masked. It is actually surprising that HEV31 and HEV4 were both able to score as neutralizing in this stringent test.

In ELISA, FABS HEV31 and HEV4 competed, suggesting that their respective epitopes overlap. A totally unexpected result was the demonstration that, in contrast to HEV4, Fab HEV31 was unable to neutralize virus efficiently in the cell-culture test even though it had such potent neutralizing activity for Sar55 in the rhesus test. The differing results from the two tests did not reflect a strain difference, since substitution of Sar55 for the Akluj strain in the cell-culture test did not appreciably increase the ability of HEV31 to neutralize. It also was unlikely to be due to a difference in affinity, since HEV31 has a $K_d$ for Sar55 ORF2 of 0.8 nM, which is even higher than that of HEV4 ($K_d$ 3.3 nM), which neutralized efficiently in both tests, and it is significantly higher than that of EBL56 ($K_d$ 48.0 nM), which neutralized HEV very convincingly in the cell-culture test (Schofield et al., 2003).

At present, we do not have an explanation for the different results with HEV31 in the two neutralization tests. It is possible that HEV31 neutralization is mediated via a different pathway than that utilized by the other antibodies and that a host component such as complement plays a major role in vivo. More interestingly, it may instead be that the receptor or a co-receptor used by HEV in vivo is not identical to that on the cultured HepG2/3CA cells and that HEV31 is most affected by this difference. Additional studies will be needed to sort this out.

In summary, the assay we have developed can be used to demonstrate HEV neutralization. Broadly cross-reactive neutralizing antibodies to HEV were proved to be induced by infection with any one of the four mammalian genotypes or by vaccination with a truncated recombinant capsid protein of genotype 1.

ACKNOWLEDGEMENTS

We thank Sandra Chang for secretarial help. This research was supported by the Intramural Research Program of the NIH, NIAID.

REFERENCES


of recombinant hepatitis E virus ORF2 proteins in baculovirus-infected insect cells. *Protein Expr Purif* 12, 75–84.


