Identification and Characterization of the Neutralization Epitope(s) of the Hepatitis E Virus

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INTRODUCTION

The hepatitis E virus (HEV), which is currently unclassified (Berke and Matson, 2000), is transmitted primarily by the fecal–oral route and causes epidemic and sporadic cases of hepatitis. Numerous HEV outbreaks have occurred in many developing countries, resulting in tens of thousands of people being infected. The mortality from acute HEV infection ranges from 0.5–1% for the general population to as high as 20% for infected pregnant women. Although only a few cases were diagnosed in industrialized countries, anti-HEV antibodies have been found in a significant proportion of blood donors or healthy individuals (Mast et al., 1997; Thomas et al., 1997). The reason for this relatively high seroprevalence is not well understood. One possible explanation for this observation may be subclinical HEV infections acquired from zoonotic reservoirs (Meng et al., 1997b).

HEV is a nonenveloped virus, approximately 27–34 nm in diameter, containing a positive-sense, single-stranded RNA genome of approximately 7.5 kb in length. The viral genome contains three partially overlapping open reading frames (ORFs), with ORF1 encoding nonstructural proteins (pORF1), ORF2 encoding a putative 660-amino-acid (aa) capsid protein (pORF2), and ORF3 encoding a small protein (pORF3) of uncertain function (Tam et al., 1991). Full-length genomes of several HEV strains from Asia and North America have been sequenced (Tam et al., 1991; Huang et al., 1992; Erker et al., 1999; Wang et al., 2000). Phylogenetic analysis based on the full-length sequences suggests the presence of four distinct genotypes represented by the Burma strain, Mexico strain, US-1 strain, and the new Chinese variant T1 (Erker et al., 1999; Wang et al., 2000). More HEV genotypes and subtypes have been identified using sequences derived from small PCR fragments (Schlauder et al., 2000). However, the antigenic structure of this virus has not been thoroughly studied, although several antigenic regions of diagnostic relevance were identified from small PCR fragments derived from the US-1 strain (Li et al., 1997; Khudyakov et al., 1994a, 1994b, 1999). In addition, recombinant proteins (Yarbough et al., 1991; Li et al., 1997). The HEV neutralization epitope(s) has not yet been identified.

One well-established approach to define neutralization epitopes is by an analysis of virus mutants resistant to neutralizing monoclonal antibodies. This approach was successfully applied to many viruses, for example, picornaviruses (Diamond et al., 1985). However, it is not possible to identify neutralization epitopes for each virus by this approach. The development of highly sensitive and specific diagnostic tests is not well understood. One possible explanation for this relatively high seroprevalence is the presence of four distinct genotypes. Thus, recombinant proteins constructed in this study may be considered as potential candidates for the development of an HEV subunit vaccine as well as for the development of highly sensitive and specific diagnostic tests.
applicable to HEV because of the absence of characterized HEV mutants, although two neutralizing monoclonal antibodies were recently prepared by phage display (Schofield et al., 2000). Another approach is by means of detecting neutralizing antibodies against numbers of synthetic peptides and recombinant proteins. Such large-scale testing can be performed only using an in vitro neutralization assay. However, the lack of an efficient cell culture system capable of supporting HEV propagation has hampered the development of such a traditional assay. Recently, a PCR-based in vitro HEV neutralization assay was developed (Meng et al., 1997a) on the basis of our previous observation that the infection of HEV to a human hepatocarcinoma cell line, PLC/PRF/5, can be detected by PCR over 3 weeks in vitro (Meng et al., 1996). Antibodies against the HEV recombinant C2 protein comprising the C-terminal two-thirds (225–660 aa) of the HEV Burma pORF2 (Purdy et al., 1992) were found to neutralize the HEV Burma, Mexico, and Pakistan strains by using the in vitro neutralization assay (Meng et al., 1998). This finding suggested that the HEV neutralization epitope(s) might be located within the C-terminal part of its pORF2.

In the present study, 51 overlapping 30-mer synthetic peptides spanning the entire C2 protein and 31 overlapping recombinant proteins of different sizes derived from the entire Burma pORF2 were prepared and used to immunize mice. All immune sera were tested by the in vitro neutralization assay. Antibodies obtained against recombinant proteins containing region 452–617 aa demonstrated specific HEV neutralizing activity. These data establish for the first time that the minimal size fragment of the HEV pORF2 that can efficiently model the neutralization epitope(s) is 166 aa in length and is located at position 452–617 aa. These recombinant proteins that efficiently model the HEV neutralization epitope(s) may be considered as candidates for the development of an HEV subunit vaccine as well as for the development of highly sensitive and specific diagnostic tests.

RESULTS

Neutralizing activity of antibodies against synthetic peptides

Fifty-one immune sera against BSA-conjugated HEV synthetic 30-mer peptides spanning the region at position 221–660 aa of the Burma HEV pORF2 (Khudyakov et al., 1999) were tested by both enzyme immunoassay (EIA) and the in vitro neutralization assay. All of the serum samples were immunoreactive to the BSA carrier protein, suggesting that mice developed immune responses to these conjugated antigens. However, antibodies immunoreactive with the corresponding synthetic peptides were identified in only 31 of the 51 (61%) serum samples. These immunoreactive samples appeared to cluster into five groups: group 1 (4 peptides derived from region 221–271 aa), group 2 (6 peptides derived from region 301–363 aa), group 3 (10 peptides derived from region 381–471 aa), group 4 (10 peptides derived from region 483–601 aa), and group 5 (1 peptide derived from region 631–660 aa). However, when the 51 immune sera were tested by the in vitro neutralization assay, none of them demonstrated neutralizing activity to either the HEV Burma or Mexico strain (data not shown). Since antibody mixtures have been shown to enhance an antibody’s neutralizing activity (Martinez and Melero, 1998), antisera from each group were pooled and retested by the in vitro neutralization assay. None of the pooled sera demonstrated any neutralizing activity (data not shown). Therefore, although antibodies obtained by immunization of mice with HEV synthetic peptides could be detected by EIA, none of the antisera, either alone or pooled, neutralized HEV in the in vitro neutralization assay. These observations suggested that the HEV neutralization epitope(s) is distinctly different from epitopes eliciting

<table>
<thead>
<tr>
<th>HEV–GST fusion protein</th>
<th>Location (aa)</th>
<th>Length (aa)</th>
<th>Predicted size (kDa)</th>
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<tr>
<td>pA1</td>
<td>1–103</td>
<td>103</td>
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<td>103</td>
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<td>+</td>
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<td>234</td>
<td>51.7</td>
<td>+</td>
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<tr>
<td>pB166</td>
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<td>166</td>
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</table>
EIA-detectable antibodies and indicated that the neutralization epitope(s) could not be modeled with short linear synthetic peptides.

**HEV–GST fusion proteins**

Thirty-one overlapping PCR fragments comprising different regions of the HEV Burma ORF2 sequence were separately amplified and cloned with the prokaryotic expression vector pGEX-4T-2. The presence of corresponding fragments in each clone was confirmed by DNA sequencing. After isopropyl-β-D-thiogalactopyranoside (IPTG) induction, hybrid HEV–GST proteins of the expected molecular weight (Table 1) were expressed for each clone as shown in Fig. 1. The majority of these proteins were insoluble and produced inclusion bodies. Some proteins such as pA1, pA2, pA3, pA4, pA5, pA6, pA7, pA8, pA9, pA10, pA11, pA12, pA13, pA14, and pA15; Lanes 16–19, ~100-aa-long proteins (pF1, pF2, pF3, and pF4); Lanes 20–26, pF4 derivatives by N-terminal truncation (pN309, pN336, pN384, pN393, pN421, pN452, and pN499); Lanes 27–30, pF4 derivatives by C-terminal truncation (pC617, pC580, pV540, and pC507); Lane 31, pB166; Lane 32, GST; Lane 33, protein molecular weight markers (Low range, Bio-Rad, Hercules, CA).

Neutralizing activity of antibodies against proteins pA1–pA15 and pF1–pF4

Fifteen immune serum samples against ~100-aa-long recombinant proteins (pA1–pA15) and four immune serum samples against ~400-aa-long recombinant proteins (pF1–pF4) were first tested for anti-GST by EIA. All serum specimens were immunoactive against GST, thus confirming that all of the recombinant proteins elicited antibodies in immunized mice. However, when the in vitro neutralization assay was performed with both the HEV Burma and Mexico strains, the serum samples against each of the ~100-aa-long recombinant proteins as well as three of the ~400-aa-long proteins such as pF1 (1–417 aa), pF2 (113–507 aa), and pF3 (189–580 aa) did not show any neutralizing activity (Figs. 2B and 2C). Only antisera against pF4, comprising the C-terminal part (274–660 aa) of pORF2, neutralized both the Burma and Mexico strains in the in vitro neutralization assay (Fig. 2C).

To further study the neutralizing activity of antibodies against recombinant proteins, three pools of serum specimens were prepared. One pool was composed of serum specimens obtained by immunization with recombinant protein pA8–pA15, the second by immunization with protein pF2 and pA12–pA15, and the third with pF3 and pA14–pA15. The in vitro neutralization assay demonstrated that none of the pools was capable of neutralizing either the Burma or the Mexico strain, although the corresponding sequences of these protein combinations were equal to or even longer than that of pF4 (274–660 aa). These findings suggested that the HEV neutralization epitope(s) can be modeled only with the pF4 protein, which is 387 aa in length and is located at the C-terminal part of the pORF2. None of the short ~100-aa fragments, or the other ~400 aa fragments lacking the C-terminal sequence of the pORF2, or combinations of these proteins could model this epitope(s).

Neutralizing activity of antibodies against truncated pF4 proteins

To finely map the HEV neutralization epitope(s), an additional 12 immune serum samples prepared against...
recombinant proteins obtained by truncating pF4 from the N- and/or C-termini were tested by EIA and by the in vitro neutralization assay. Each of the mouse antisera immunoreacted with GST by EIA, indicating that the immunized mice generated antibodies to these truncated HEV–GST fusion proteins. Interestingly, as shown in Fig. 2D, immune sera against proteins pN309, pN336, pN364, pN393, pN421, and pN452, which were derived from pF4 by truncating from the N-terminus at positions 309, 336, 364, 393, 421, or 452 aa, respectively, demonstrated HEV neutralizing activity similar to antibodies against pF4. However, when immune sera against a protein truncated at position 499 aa, designated pN499, were tested, neutralizing activity was not observed. This finding suggested that the region at position 452–499 aa plays an essential role in modeling the HEV neutralization epitope(s). Additionally, when serum samples from mice immunized with the other pF4 derivatives, pC617, pC580, pC540, and pC507, obtained by truncation from the C-terminus at positions 617, 580, 540, or 507 aa, respectively, were tested by the in vitro neutralization assay, only anti-pC617 neutralized both HEV strains (Fig. 2E). This finding demonstrated that the region between 617 and 660 aa of the pORF2 is not involved in modeling the HEV neutralization epitope(s), whereas the region between 580 and 617 aa is essential for eliciting the HEV neutralizing activity.

Finally, when antisera prepared against a 166-aa-long protein (pB166), which was derived from pF4 by truncating from the both N- and C-termini at positions 452 and 617 aa, were tested by the in vitro neutralization assay, neutralizing activity against both Burma and Mexico HEV strains was observed (Fig. 2F). Thus, the smallest region identified in the present study that can efficiently model the HEV neutralization epitope(s) is 166 aa in length and is located at position 452–617 aa of the HEV pORF2.

Cross-neutralization of different HEV strains with anti-pB166

As shown above, pB166 is the minimal size protein capable of modeling the HEV neutralization epitope(s). Given its small size, pB166 may contain a smaller array of antigenic epitopes compared to larger proteins. As a result, pB166 may be expected to be less prone to nonspecific immunoreactivity than larger proteins. Because of this hypothesis, pB166 was studied in more detail by conducting additional quantitative assays to compare the neutralizing activity of antibodies against pB166 with HEV strains from Burma, Pakistan, Morocco, Mexico, and the United States representing the different HEV genotypes and subtypes. Twofold dilutions starting from 1:10 of the immune serum against pB166 were prepared and mixed with 100 cell culture infectious doses of each HEV strain. After incubation at 37°C for 1 h, these mixtures were inoculated onto PLC/PRF/5 cells. Neutralization was determined by PCR as described under Materials and Methods. The end-point neutralization titers of anti-pB166 for the Burma, Mexico, and US-1 strains were 1:640 and that for the Pakistan strain was 1:1280. The HEV Morocco strain, however, was not reproducibly neutralized with anti-pB166 diluted beyond 1:20. These observations demonstrated that antibodies against pB166 cross-neutralize the different geographic HEV strains, although with variable efficiency, suggesting that a common neutralization epitope(s) may exist within the different HEV genotypes and subtypes.
DISCUSSION

An in vitro neutralization assay has traditionally been defined by the absence of virus-induced cytopathic effect or the reduction of viral plaques in cell culture. However, many other direct and indirect markers have been used, such as a reduction in the number of infected cell foci visualized by hemadsorption, immunofluorescence, immunoenzyme assay, or a reduction in the presentation of virus-specific antigens detected by hemagglutination or EIA. As a new marker of neutralization, many laboratories utilize the detection of viral DNA or RNA molecules in inoculated cells by PCR. This has been particularly important for these viruses that remain refractory to propagation in cell culture (Haraguchi et al., 1994; Shimizu et al., 1994; Meng et al., 1996, 1997a; Zibert et al., 1997; White et al., 1998; Yang et al., 1998). The exact mechanisms responsible for virus neutralization are not well understood and may involve blocking of attachment and entry, prevention of fusion, and uncoating. Nevertheless, all the neutralization tests are based on the assumption that neutralizing antibodies block viral replication, regardless of the stage at which this effect occurs. Recently, the mechanism of blocking viral attachment and entry, the first step in viral replication, has been successfully applied to several rapid PCR-based neutralization assays for the detection of neutralizing antibodies against hepatitis C virus (Shimizu et al., 1994; Zibert et al., 1997), human immunodeficiency virus (Yang et al., 1998), and human T-cell leukemia virus (Haraguchi et al., 1994).

We previously developed a similar neutralization assay for HEV based on the identical neutralization mechanism (Meng et al., 1997a). This assay was shown to be highly specific and sufficiently sensitive to evaluate the neutralizing activity in various anti-HEV serum specimens (Meng et al., 1998). The neutralization results obtained by the rapid assay were identical to those obtained by an assay in which the neutralization was determined after incubation of inoculated cells for 7 days (Meng et al., 1996, 1997a).

In this paper, this rapid in vitro HEV neutralization assay was applied to study the HEV neutralization epitope(s). In accordance with the sequence of the C2 protein (225–660 aa), 51 overlapping 30-mer synthetic peptides spanning the Burma strain pORF2 at position 221–660 aa were used in an attempt to model the neutralization epitope(s). However, none of these peptides elicited neutralizing antibodies. Although synthetic peptides derived from other hepatitis viruses such as hepatitis A virus (Emini et al., 1985), hepatitis B virus (Neurath et al., 1986), and hepatitis C virus (Shimizu et al., 1996) have been shown to elicit neutralizing antibodies, these synthetic peptides did not efficiently model the neutralization epitopes of these viruses and, as a result, have not been used in vaccine development. In general, synthetic peptides form only linear epitopes that have low intrinsic immunogenicity. Thus, they are not efficient immunogens for eliciting a neutralizing antibody response (Yewdell and Bennink, 1997). The failure of modeling the HEV neutralization epitope(s) with the synthetic peptides suggests that this epitope(s) is not a linear epitope(s).

Many neutralization epitopes are conformation-dependent discontinuous epitopes formed by residues derived from different regions of the unfolded polypeptide chain when the protein folds (Yewdell and Bennink, 1997). In such cases, the neutralization epitopes can be modeled only with protein fragments that contain all of the necessary information to direct the correct folding. As shown in this study, only proteins containing the region 452–617 aa are able to elicit an HEV neutralizing immune response. Deletion of 48 aa (452–499 aa) or more from the N-terminus or 38 aa (617–580 aa) or more from the C-terminus of the region 452–617 aa led to the inability of the protein to elicit neutralizing antibodies. Interestingly, Li et al. (1997) made an observation that the full-length pORF2 and a protein containing the N-terminal region at 1–110 aa strongly immunoreacted with HEV acute-phase sera by immunoblot analysis, but were poorly immunoreactive with convalescent sera. Conversely, the recombinant protein containing the C-terminal region at 394–660 aa strongly immunoreacted with both acute- and convalescent-phase sera. Any extension of this protein toward the N-terminus or any truncation from the N-terminus of this protein caused a noticeable loss of immunoreactivity with convalescent-phase sera. Since immunoblot analysis does not specifically detect the neutralizing immune response, there is no direct evidence that the same antibodies were detected by Li et al. (1997) and in the present study. Nevertheless, there is a striking similarity in the conformational dependence of antibody binding by different recombinant HEV antigens. In addition, Schofield et al. (2000) recently performed a radioimmunoprecipitation study with two HEV neutralizing monoclonal antibodies and a set of five C-terminally truncated pORF2 of the HEV Pakistan strain. The monoclonal antibodies precipitated the recombinant protein corresponding to 112–607 aa but did not precipitate those terminating at or prior to 578 aa. Although they did not try to determine the N-terminal binding position for the monoclonal antibodies, the C-terminal position they determined is very close to that determined in the present study.

As shown in the present study, all proteins containing the HEV neutralizing activity detected by the in vitro neutralization assay were also specifically immunoreactive with serum specimens obtained from a cynomolgus macaque experimentally infected with the HEV Pakistan strain. This finding suggests that the immune response elicited against these proteins after immunization patterns to some extent the immune response caused by HEV infection. Furthermore, as shown previously (Purdy et al., 1993) the recombinant C2 antigen (225–660 aa of
the HEV Burma ORF2 protein), which contains the primary structure of all of the neutralizing recombinant proteins obtained in this study, when used as immunogen protected cynomolgus macaques from infection following HEV challenge. Taking into consideration that antibodies obtained against this C2 antigen have been shown to neutralize different HEV strains in the in vitro assay used in the present work (Meng et al., 1998) and that all neutralizing HEV proteins used in this study as well as the C2 antigen are derived from the same HEV Burma strain, it can be hypothesized that the HEV neutralizing activity detected by the in vitro neutralization assay is cognate to the HEV protective immune response in the experimental animal models.

This paper describes the immunoreactivity of proteins shorter than the full-size pORF2. Truncating the N-terminal 451 aa (1–451 aa) and/or the C-terminal 43 aa (618–660 aa) from the full-size pORF2 did not affect the function of the neutralization epitope(s). Conversely, our previous results demonstrated that a full-length pORF2 expressed in a baculovirus system was less efficient in eliciting neutralizing antibodies than the truncated form, such as the C2 protein (Meng et al., 1998). Additionally, as was recently shown, nonhuman primates immunized with a full-length recombinant pORF2 expressed in *Escherichia coli* were not protected against challenge with HEV, despite the fact that these animals elicited high titers of immunoblot-detectable antibodies (Panda et al., 1997). Collectively, these observations suggest that the HEV neutralization epitope(s) may be modeled with variable efficiency by pORF2 recombinant proteins of different sizes and that the presence of the N-terminal domain might have an adverse effect on modeling this epitope(s) by these proteins.

Recently, several research groups reported that the HEV ORF2 protein formed virus-like particles (VLPs) when expressed in insect cells (Tsarev et al., 1997; Zhang et al., 1997; Li et al., 2000b). Since virions are the target for neutralizing antibodies it seems logical to assume that VLPs, which model virion macrostructure, would also efficiently model the neutralizing epitope(s) and, therefore, efficiently elicit neutralizing antibodies. In fact, several groups have published experimental evidence that HEV VLPs are very potent immunogens affording protection against both homologous and heterologous challenge in nonhuman primates (Tsarev et al., 1994, 1997). The strategy presented in this study and noted by other researchers (Purdy et al., 1993; Li et al., 2000a; Im et al., 2001) allows for the efficient modeling of the HEV neutralizing antigenic epitope(s) without the requirement for modeling the macrostructure into VLP. Immunization with the recombinant proteins described in this paper induced synthesis of antibodies with anti-HEV neutralizing activity detected by the in vitro neutralization assay. Similar proteins have been used to successfully protect nonhuman primates against HEV infection (Purdy et al., 1993; Im et al., 2001). These observations strongly suggest that this alternative strategy should be considered for the development of promising HEV vaccine candidates.

The HEV genome is rather heterogeneous. Therefore, the virus can be classified into several genotypes and subtypes (Schlauder et al., 2000; Wang et al., 2000). However, the effect of sequence heterogeneity on the HEV antigenic properties, especially on the neutralization epitope(s), has not been thoroughly investigated. It was reported that baculovirus expressed recombinant proteins containing the pORF2 region of 112–660 aa from the HEV Pakistan strain (genotype 1, subtype 1b) or the Burma strain (genotype 1, subtype 1a) induced protective immune responses to a heterologous Mexico strain (genotype 2) challenge in nonhuman primates (Tsarev et al., 1997; Yarbough et al., 1997). Also, antibodies against the C2 protein, which contains the Burma pORF2 fragment of 225–660 aa, efficiently neutralized the HEV Burma, Pakistan, and Mexico strains, but not the Morocco strain (genotype 1, subtype 1c) in the in vitro neutralization assay (Meng et al., 1998). In the present study, this observation was confirmed by a quantitative cross-neutralization assay using the immune sera against the small protein, pB166. This sample neutralized the Burma, Pakistan, Mexico, and US-1 strains at the end-point titers of 1:640 to 1:1280, whereas the Morocco strain was neutralized at a lower titer of 1:20. This is the first experimental evidence that the HEV US-1 strain (genotype 3) can be cross-neutralized with antibodies against the HEV Burma strain. The reason for the lower efficiency of neutralization of the HEV Morocco strain noted previously (Meng et al., 1999) and confirmed in the present study is not clear. One probable explanation is that because the Morocco strain was obtained from a pool of fecal specimens collected from 15 HEV-infected individuals from an outbreak of hepatitis E in Morocco, it is possible that this pool contains one or more neutralization escape mutants.

In conclusion, this study has shown that the region at position 452–617 aa of pORF2 is the minimal size fragment capable of efficiently modeling the HEV neutralization epitope(s). Antibodies obtained by immunization of mice with pB166, a 166-aa-long recombinant protein corresponding to the identified region, can cross-neutralize several different geographic HEV strains in the in vitro neutralization assay. We believe the data presented in this paper should be taken into account in the development of HEV vaccines. In addition, we believe that further characterization of the HEV neutralization epitope(s) will be very helpful in identifying HEV neutralization escape mutants, as has been shown with the "a" determinant of the hepatitis B virus (Seddigh-Tonekaboni et al., 2000).
MATERIALS AND METHODS

Cell culture

PLC/PRF/5 was grown in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Laboratory, Int., Logan, UT) and incubated at 37°C with 5% CO2. For the in vitro neutralization assay, trypsinized cells were seeded into 24-well, flat-bottom culture plates at a concentration of 105 cells per well and incubated to form cell monolayers.

Virus stocks

Inocula containing the HEV Burma, Pakistan, Morocco, and Mexico strains were previously described (Meng et al., 1998). The HEV US-1 strain was obtained from a fecal sample collected from a 62-year-old white male with acute hepatitis without a history of recent travel outside the United States (Kwo et al., 1997). This inoculum was kindly provided by K. McCaustland [Hepatitis Branch, Centers for Disease Control and Prevention (CDC), Atlanta, GA] and prepared as previously described (Meng et al., 1998).

Synthetic peptides

Fifty-one overlapping 30-mer peptides spanning the C2 protein sequence were previously described (Khudiyakov et al., 1999).

Construction of HEV recombinant plasmids

Thirty-one HEV recombinant plasmids were constructed by cloning PCR fragments with pgEX-4T-2 vector (Pharmacia Biotech Inc., Piscataway, NJ). All PCR fragments were amplified from a recombinant plasmid containing the whole HEV Burma strain ORF2 sequence (kindly provided by Dr. S. Kamili, Hepatitis Branch, CDC). Primers used for PCR amplification were selected based on the Burma strain sequence (Tam et al., 1991) and modified to contain a BamHI or XhoI restriction site to facilitate cloning (not shown). PCR was performed using the Expand High Fidelity PCR System (Boehringer Mannheim, GmbH, Mannheim, Germany). PCR products were purified with the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA). Both purified PCR products and pgEX-4T-2 vector were digested with BamHI and XhoI (Boehringer Mannheim) at 37°C overnight, ligated using T4 DNA ligase (Pharmacia Biotech) at 16°C overnight, and then used to transform E. coli competent JM109 cells (Promega, Madison, WI). Recombinant plasmids were recovered from transformants by using the Wizard Miniprep DNA Purification system (Promega). The presence of insert was confirmed by PCR using two primers with the sequences, 5'-CAG GGC TGG CAA GCC AC and 5'-CGT CAT CAC CGA AAC GC, derived from regions flanking the multiple cloning site of pgEX-4T-2. The primary structure of the inserts was finally confirmed by DNA sequencing with a Model 373 or 377 automated DNA sequencer (ABI, Foster City, CA).

Expression of HEV–GST fusion proteins

E. coli JM109 cells transformed with the recombinant plasmids were grown at 37°C overnight in Luria broth (LB) medium containing 50 μg/ml ampicillin. The overnight culture was diluted 20 times with fresh LB medium containing the same concentration of ampicillin and grown at 37°C for 3 to 4 h until an optical density value of 0.6–1.0 at 600 nm was reached. The gene expression was induced by adding IPTG (Sigma Chemical Co., St. Louis, MO) into the culture to a final concentration of 1 mM. After 4 h of incubation at 37°C with constant shaking, the cells were pelleted by centrifugation at 6000 g for 15 min at 4°C and then resuspended with 3 ml of lysis buffer (50 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl) for each gram of packed cells. The suspension was incubated on ice for 30 min with a final concentration of 0.2 mM phenylmethylsulfonyl fluoride (Sigma) and 0.5 mg/ml of lysozyme (Sigma). Then, 4 mg of deoxycholic acid was added per gram of E. coli cells while stirring continuously at room temperature for 5 min. The lysate was incubated with 20 U/ml of DNase (Boehringer Mannheim) at room temperature until it was no longer viscous and centrifuged at 10,000 g for 20 min at 4°C. The supernatant was transferred to a fresh tube and purified with Bulk and Redipack GST Purification Modules (Pharmacia Biotech). The pellet containing insoluble HEV–GST fusion protein was washed completely, resuspended and homogenized with phosphate-buffered saline (PBS), and stored in aliquots at −70°C.

Immunoblot analysis of HEV recombinant proteins

Aliquots of each homogenized HEV–GST fusion protein were separated by electrophoresis on precast 12% sodium dodecyl sulfate (SDS)–polyacrylamide gels (Bio-Rad, Richmond, CA) followed by blotting onto nitrocellulose membranes (Bio-Rad). The nitrocellulose membranes were incubated overnight with blocking buffer containing 10% normal goat serum, 1% bovine serum albumin (BSA), and 0.05% Tween 20 in 0.01 M PBS and then incubated for 1 h with serum samples collected from a cynomolgus macaque experimentally infected with the HEV Pakistan strain (Pillot et al., 1995) diluted 1:100 in blocking buffer. The membranes were rinsed three times with wash buffer (PBS with 0.05% Tween 20) and incubated for 1 h with affinity-purified goat anti-human immunoglobulin G (IgG, Pierce) conjugated with horseradish peroxidase diluted 1:6000 in blocking buffer. After three washes, color development was carried out with 3,3′-diaminobenzidine as substrate (Bio-Rad).
Preparation of immune sera

All synthetic peptides were conjugated with BSA by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride coupling methods using a commercially available kit (Pierce, Rockford, IL). Each of the BSA-conjugated peptides and the HEV–GST fusion proteins was emulsified with adjuvant (TiterMax; CyRx, Atlanta, GA) and used to immunize a group of three or four female Hsd NIH mice that were 6–9 weeks old. Mice were inoculated subcutaneously at two sites on the back with a total of 100 μl of the emulsion containing 50 μg of the conjugated peptide or the fusion protein. Four weeks later, the mice were boosted with an intraperitoneal injection of 10 μg of the same peptide or protein diluted in 100 μl of PBS. Seven days later, the mice were bled from the heart. The immune sera obtained from each group of mice were pooled together and inactivated by heating at 60°C for 30 min. Aliquots were prepared and stored at −70°C. Immune sera against BSA and GST were prepared using the same procedure.

Enzyme immunoassay

The protocol used for detecting antibodies against HEV synthetic peptides was previously described (Khudyakov et al., 1999).

In vitro PCR-based neutralization assay

This assay has been previously described (Meng et al., 1997a, 1998). Briefly, approximately 100 cell culture infectious doses of an HEV inoculum diluted in 100 μl of Hanks’ solution were mixed with 100 μl of immune sera at a dilution of 1:10. After incubation at 37°C for 1 h, the mixture was inoculated onto a cell monolayer of PLC/PRF/5. After adsorption for 2 h at 37°C, the cells were washed three times with Hanks’ solution followed by immediate RNA extraction with TRIzol reagent (Gibco BRL) according to the manufacturer’s instructions followed by reverse transcription and nested PCR using a set of universal HEV PCR primers. The external primers were JM-2 (5′-CCG ACA GAA TTG ATT TCG TCG GC) and JM-5 (5′-CCG TAA GTG GAC TGG TCG TAC TC). The internal primers were JM-3 (5′-GGT GTC TCG GCC AAT GGC GAG CC) and JM-4 (5′-TCG GCG GCG GTG AGA GAG AGC CA). Both the first-round and the second-round amplifications were carried out according to the following cycling program: denaturation at 94°C for 45 s, annealing at 60°C for 20 s, and extension at 72°C for 30 s, for 30 cycles. Amplicons were separated by agarose gel electrophoresis with size markers and visualized by ethidium bromide fluorescence. Neutralization was determined by the absence of detectable HEV RNA in the inoculated cell culture. A normal mouse serum control, anti-BSA or anti-GST serum control, virus control, and uninoculated cell control were processed for detection of HEV RNA at the same time.

REFERENCES

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