Comparative characterization of antigenic epitopes in the immunodominant region of the protein encoded by open reading frame 3 in Burmese and Mexican strains of hepatitis E virus

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To analyse the effect of strain-specific sequence variation on the antigenic properties of the protein encoded by the open reading frame 3 (ORF 3) of hepatitis E virus (HEV), two sets of short overlapping peptides spanning amino acids 91 to 123 of this protein from Burmese and Mexican strains were synthesized and tested with sera obtained from outbreaks of enterically transmitted non-A, non-B hepatitis in three different regions of the world (Mexico, Turkmenistan and Kenya). The data suggest strain-specific variation in the antigenic reactivity of the ORF 3 protein. The C-terminal region of this protein contains several antigenic epitopes located in the most variable positions. Individual sera were found to interact with different groups of epitopes from each set of peptides. The antigenic epitopes of the Mexican strain appear to be less conformation-dependent than those of the Burmese strain. The most immunoreactive epitope of the ORF 3 protein from the Mexican strain was localized at amino acid positions 95 to 101. The ORF 3 protein of the Burmese strain contains an immunodominant epitope at amino acid positions 112 to 117. Some of these short peptides may be useful for the development of a diagnostic assay to discriminate between the Burmese and Mexican strains.

Hepatitis E virus (HEV) is an agent of enterically transmitted non-A, non-B hepatitis (ET-NANB) (Bradley, 1990; Reyes et al., 1990, 1991a), which is a serious problem in many developing countries (Bradley, 1990; Wright, 1990). Recent success in cloning and sequencing of the HEV genome (Tam et al., 1991; Tsarev et al., 1992; Aye et al., 1993) has allowed the elucidation of the genetic organization of this virus. Three open reading frames (ORFs) have been identified (Tam et al., 1991). The non-structural proteins are encoded by ORF 1 (Reyes et al., 1991b), whereas ORF 2 is probably responsible for the synthesis of structural proteins. ORF 3 encodes a protein of unknown function(s), of 123 amino acids (Tam et al., 1991). The primary structure of proteins encoded by all three ORFs is similar within the Burmese group with rare amino acid changes in proteins encoded by ORFs 2 and 3, whereas the polypeptide encoded by ORF 1 contains one hyper-variable domain (Tsarev et al., 1992). Several antigenic epitopes have been mapped in these proteins (Yarbough et al., 1991; Kaur et al., 1992; Khudyakov et al., 1993). The primary structure of the antigenic region located at the C terminus of the ORF 3 protein has been shown to be highly conserved within the Burmese group (Tam et al., 1991; Tsarev et al., 1992; Aye et al., 1993; Bi et al., 1993) and significantly different from the Mexican strain (Yarbough et al., 1991). Only 78% sequence identity has been found between the C-terminal regions of ORF 3-encoded proteins of the HEV Burmese and Mexican strains (Yarbough et al., 1991). This variation in primary structure has been demonstrated to change the antigenic properties of the region (Yarbough et al., 1991; Khudyakov et al., 1993). It has also been shown that a short fragment of the ORF 3 protein, derived from the Burmese strain, did not react with serum samples from cynomolgus macaques infected with the Mexican strain. Conversely, recombinant protein derived from the Mexican strain did not react with serum specimens from macaques infected with the Burmese strain (Yarbough...
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To analyse the effect of sequence variability on the antigenic characteristics of the C-terminal region of the HEV ORF 3 protein, a set of overlapping synthetic peptides was prepared. Synthetic peptides containing hexamer sequences which overlap by five amino acids corresponded to amino acids 91 to 123 of the ORF 3 protein from the Mexican and Burmese strains of HEV. Lysine was added to the N terminus of each hexamer and two residues of glycine to the C terminus. Addition of these amino acids was designed to improve the efficiency of chemical synthesis because the sequence is saturated with prolines. Also, the addition of these amino acids would provide more uniform adsorption to microtitre wells and solubility properties. Peptides corresponding to the Burmese sequence (B) or to the Mexican sequence (M) were synthesized by Fmoc-chemistry (Barany & Merrifield, 1980) on an ACT Model MPS 350 multiple peptide synthesizer (Advanced Chemtech). After characterization by amino acid analysis, HPLC and capillary electrophoresis, peptides were used directly in an enzyme immunoassay (EIA) as described elsewhere (Khudyakov et al., 1993). Briefly, synthetic peptides were adsorbed to microtitre wells (Immulon II, Dynatech) and allowed to interact with human anti-HEV or guinea-pig anti-peptide sera diluted 30-, 50- or 100-fold for 1 h at 37 °C. The antigenic activity of peptides was identified using affinity.

Fig. 1. Comparison of Mexican and Burmese sequences of the C-terminal region of the ORF 3 protein (Yarbough et al., 1991). Locations of large peptides 5, 6, 28 and 29 (Khudyakov et al., 1993), as well as those synthesized in this study (hexamers 1 to 28), are shown.

Fig. 2. Interaction of short peptides from the Mexican set (M peptides) and from the Burmese set (B peptides) with guinea-pig antisera raised to large peptides 5 (■), 6 (□), 28 (◆) and 29 (□) (Fig. 1).
chromatography-purified antibodies to human or guinea-pig IgG conjugated to horseradish peroxidase (Tago). Peptides were evaluated as immunoreactive if the P:N ratio was greater than 3:0, where P represents the A value at 490 nm obtained with an anti-HEV-positive specimen and N represents the A value of negative controls.

Initial experiments were conducted with guinea-pig sera containing antibodies to two pairs of overlapping synthetic peptides: 5 and 6, 28 and 29 (Khudyakov et al., 1993). These peptides represent the C-terminal sequences of the ORF 3 protein belonging to the HEV Burmese and Mexican strains, respectively (Fig. 1). We found that antibodies specific to peptides from the Mexican HEV strain (anti-28 and -29) did not cross-react with peptides from the Burmese HEV strain (peptides 5 and 6, and peptides of series B). Similarly, anti-5 and anti-6 antibodies did not react with peptides 28 and 29, and peptides of series M. Thus, these data confirm the observed strict strain-specific immunoreactivity of the C-terminal region of the ORF 3 protein (Yarbough et al., 1991). Guinea-pig anti-peptide 28 and anti-peptide 29 sera strongly bind peptides M2 (NQPGHL) and M3 (QPGHLA), and M19 (PLPPVA), respectively (Fig. 2b). Peptides 5 and 6 from the Burmese HEV strain, and peptides 28 and 29 from the Mexican HEV strain overlap in the most conserved region of the C-terminal sequence (Fig. 1). However, the antigenic epitopes of these peptides are located in the less conserved regions and their positions overlap but are not identical (Fig. 2). The data suggest that the changes in the amino acid sequences found in the ORF 3 proteins of the Burmese and Mexican HEV strains influence the cross-immunoreactivity and the location of the epitopes as modelled with antibodies against synthetic peptides.

Human anti-HEV-positive sera were obtained from outbreaks of ET-NANB in Turkmenistan in 1985 (M. O. Favorov, unpublished data), Mexico in 1986 (Centers for Disease Control, 1987) and Kenya in 1991 (Centers for Disease Control, unpublished). The anti-HEV status of these serum specimens was confirmed by a recently developed Western blot analysis (Favorov et al., 1992) and by a peptide-based EIA (Favorov et al., 1994). Sera obtained from Turkmenistan and Kenya have been shown to be antigenically related to the HEV Burmese
group by immunoreactivity with synthetic peptides derived from the HEV Mexican or Burmese strain proteins (Khudyakov et al., 1993). Geographically, Turkmenistan is located near the regions from which all members of the HEV Burmese group have been isolated (Tam et al., 1991; Tsarev et al., 1992; Aye et al., 1993; Bi et al., 1993) and, therefore, their relatedness to the Burmese group was anticipated.

A total of 37 anti-HEV-positive sera obtained from HEV outbreaks (Mexico, n = 14; Turkmenistan, n = 16; Kenya, n = 7) were used to compare the immunoreactivity of peptides. Negative sera (n = 7) were collected from healthy people residing in non-endemic regions. Of 37 anti-HEV positive sera, 11 were non-reactive with peptides from the Mexican set, and 13 sera were non-reactive with peptides from the Burmese set. In general, the sera from Turkmenistan (Fig. 3) and from Kenya (data not shown) are less reactive with both the M or B synthetic peptides than sera obtained from an outbreak in Mexico (Fig. 3). Similarly, synthetic peptides based on the sequence of the Mexican HEV strain are relatively more immunoreactive than peptides containing the Burmese sequence (Fig. 4). This observation may suggest that the Mexican sera used in this study are more closely related to the M peptides than the Turkmenian sera are related to the B peptides. However, data presented in Fig. 3 indicate that despite the different pattern of reactivity of the Mexican or Turkmenian sera with synthetic peptides from both sets, a relatively higher proportion of the Mexican sera react with the M or B peptides compared to the Turkmenian sera. In addition, the C-terminal region of the ORF 3-encoded protein is almost identical among all members of the HEV Burmese group, with only a single example of an amino acid change for one HEV isolate (Tam et al., 1991; Tsarev et al., 1992; Aye et al., 1993; Bi et al., 1993). Taking these facts into consideration, we favour the hypothesis that the antigenic epitopes are more properly modelled in hexamers containing the Mexican sequence than the Burmese sequence, and antibodies elicited to these epitopes in Mexican sera react better with epitopes modelled in synthetic peptides. Collectively, these findings suggest that the antigenic epitopes represented in the ORF 3 protein from the HEV Mexican strain are less conformation-dependent than those from the Burmese strain of HEV.

Each set contains many immunoreactive peptides. In the Mexican set, only peptides M2, M10, M13 and M26 failed to react with any anti-HEV sera. An absence of reactivity was found also for peptides B4, B7, B10, B13, B19 and B20 in the Burmese set. Taking into consideration the location of the reactive hexamers, approximately nine distinctive epitopes may be identified in both sequences and grouped by peptide numbers 1–2, 5, 8–9, 11, 14, 18, 21–22, 24–26, 28. Peptides M5, M22 and M28 from the Mexican strain, and B2, B9, B18, B22 and B26 from the Burmese strain reacted with five to eight sera out of 37 tested depending on the individual peptide (Fig. 4). Peptides M22 and M28, however, reacted with a relatively low P:N value (Fig. 4b). Peptide M5 is the most immunoreactive in the Mexican set. This finding suggests that this peptide contains the immunodominant epitope of the ORF 3 protein from the HEV Mexican strain. Alternatively, as these peptides are HEV-specific hexamers with the addition of Gly to the C terminus (see above), peptide M5 as well as peptides B5, B24 and B24 have an extended HEV-specific sequence compared with the rest of these peptides. One additional Gly residue to these peptides extends the hexamer HEV sequence to a heptamer (Fig. 1). This enlargement of the HEV-specific sequence may facilitate the expression of the antigenic property of peptide M5. Nonetheless, if this were true, it does not have as strong an effect on peptides B5, M24 or B24 as it does on peptide M5. In contrast to the Mexican strain, the Burmese strain contains the immunodominant epitope(s) in the most C-terminal region (Fig. 4). Peptide B22 reacted with a relatively large number of anti-HEV-positive sera (Fig. 4a) and demonstrated the highest P:N value (Fig. 4b). These data suggest that B22 contains the most immunoreactive epitope of the Burmese sequence. Therefore, the region at amino acid positions 91 to 123 of the ORF 3 protein from the Mexican strain of HEV contains an immunodominant antigenic activity located in the N-terminal part, and from the Burmese strain in

![Fig. 4. Reactivity of peptides from Mexican (●) or Burmese (■) sets with anti-HEV positive sera (n = 37). (a) Number of sera reactive with individual peptides in each set; (b) average P:N ratio for every peptide in each set.](image-url)
the C-terminal part. Thus, the strain-specific sequence variation affects the antigenic properties of the ORF 3 protein. Also, it is interesting to note that the M5 peptide reacted with sera obtained only from Mexico, and B22 peptide reacted with sera obtained only from Turkmenistan and Kenya (Fig. 3). In addition, there are a number of peptides in these two sets demonstrating some strain-specific reactivity.

By analysing the immunoreactivity of individual serum specimens with these two sets of synthetic peptides (B and M series), we found that any single specimen may interact with different synthetic peptides from both sets. An example of this immune interaction of antibodies from one specimen with the two sets of peptides is shown in Fig. 5. Among peptides from the Mexican set, only peptides M4, M5, M6 and M28 were found to react with antibodies in this specimen. With the exception of peptide B28, which shares sequence homology with peptide M28, different peptides were identified as immunoreactive in the Burmese set (Fig. 5). This finding suggests that this serum contains antibodies to the epitopes located in peptides 4–6, 9, 18, 21 and 28; however, depending on the strain sequence, the antibodies from this specimen recognized different epitopes in each set.

In summary, the region at amino acid positions 91 to 123 of the HEV protein encoded by ORF 3 has a very complex antigenic structure. This region contains a number of epitopes which may be affected differently by sequence variations in these two known HEV strains. We suggest that all these antigenic epitopes elicit antibodies in HEV-infected individuals. However, because of the sequence variation found for this region, different sera obtained from different individuals may have antibodies capable of recognizing only some of the epitopes that have been modelled in the two sets of peptides investigated in this study.

Thus, our data support the previous observation of strain specificity of the immune response to the C-terminal region of the ORF 3 protein from the two major strains of HEV (Yarbrough et al., 1991; Khudyakov et al., 1993). At least some of the peptides from these two sets of peptides demonstrated strain-specific reactivity. It is conceivable that with further investigation these peptides may be used for the development of a diagnostic test that will differentiate between the two known HEV strains.

References

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