

Localization of a sequential B-epitope in the VP2 protein of hepatitis A virus

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Abstract A set of synthetic peptides derived from the capsid proteins of hepatitis A virus was used to search for B-epitopes. Peptides from the 115–139 region of the VP1 protein, from the 69–99 region of the VP2 protein and peptide 137–150 from the VP3 protein were found to react with monoclonal and polyclonal anti-HAV antibodies. MAPs based on 64–80 and 66–80 fragments of VP3 were reactive as well. Peptides, their conjugates with protein carriers and MAPs were used for antipeptide antibody production. Only free peptide 69–99 from the VP2 protein caused formation of HAV binding antibodies.

Key words: Synthetic peptide; Antigenic determinant; HAV binding antibody; Antipeptide antibody; Monoclonal antibody

1. Introduction

So far there are no strong data about localization of HAV antigenic sites (B-epitopes). It is anticipated that one immunodominant neutralization site involves residues of VP3 and VP1 HAV capsid proteins [1,2]. Another neutralization domain was reported to exist in the N-terminal part of VP1 [3,4].

Recently we reported about searching for HAV B-epitopes by means of synthetic peptides [5]. We did not detect the significant binding of anti-HAV antibodies to any peptide. Our finding supported the earlier claim about the conformational nature of HAV B-epitopes [1].

This study follows on from previous work and is directed at the investigation of the HAV antigenic structure and the search for immunodominant sites on HAV capsid proteins. In the present study, the set of synthetic peptides was significantly enlarged, and monoclonal anti-HAV antibodies along with polyclonal antibodies were used.

2. Materials and methods

2.1. Synthetic peptides

Peptides were synthesized by a solid-phase method on a modernised Beckman 990 peptide synthesizer using PAM resin and HOBt esters of Boc amino acids as described earlier [5]. The synthesized peptides were

cleaved from resin and deprotected by treatment with the liquid HF containing *p*-cresol and purified by reversed-phase HPLC. The amino acid analysis of peptides was consistent with their expected compositions. The molecular masses of peptides were confirmed by FAB mass spectra.

2.2. Mice

BALB/c, CBA, C₅₇Bl₆ and F₁(CBA × C₅₇Bl₆) strains were obtained from the breeding colony at the Central nursery of laboratory animals (Russian Academy of Medical Sciences, Krasnogorsk, Russia). Mice were used at 6 to 8 weeks of age and were of either sex.

2.3. Virus

The cell culture-adapted HAS-15 strain was obtained from the Institute of Poliomyelitis and Viral Encephalitides (Moscow Region).

2.4. Anti-HAV antibodies

The IgG fraction from human reconvalescence sera was obtained from Institute of Poliomyelitis and Viral Encephalitides (Moscow Region). Monoclonal antibodies to HAV were prepared as previously described: mAb HB-1 (IgG2a) [6], mAbs B.2.6.8 and 1.2.2.2 [7]. mAbs HB-1 and B.2.6.8 were virus neutralizing.

2.5. Biotinylation of anti-HAV antibodies

Purified monoclonal and human anti-HAV IgG antibodies were dissolved in 2 ml of phosphate-buffered saline, pH 8.6 (2 mg/ml) and 100 μ l of *N*-hydroxysuccinimide-biotin in dimethylformamide (1 mg/ml) were added. The reaction was allowed to proceed overnight at 8°C or 2 h at 20°C. The reaction mixture was separated from the free *N*-hydroxysuccinimide-biotin by ultrafiltration through a YM-30 membrane (Amicon). Biotinylated antibodies were stored at –20°C in glycerol.

2.6. Peptide-specific ELISA

Binding of anti-HAV antibodies to peptides was determined by solid-phase ELISAs utilizing microtiter plates (Costar) coated overnight with peptide (20 μ g/ml) in PBS or in pH 8.9 carbonate buffer. Wells were blocked with 1% BSA for 1 h, and biotinylated anti-HAV antibodies were added for 1 h. After washing with PBS containing 0.05% Tween 20, streptavidin-biotinylated peroxidase (Amersham) was added. After 15 min of incubation plates were washed and then developed by addition of *o*-phenylenediamine in 0.1 M citrate buffer, pH 4.8 (0.5 mg/ml) and 0.002% of H₂O₂. After 0.5 h the reaction was stopped by the addition of 1 M H₂SO₄ and the optical density was measured at 492 nm using a Multiscan Reader (Labsystems, Finland). Results were considered as positive when the signal-noise ratios (S/N) were greater than 2.2.

2.7. Antipeptide antisera

Antisera were produced in mice to each of the selected peptides (free or conjugated with KLH or Ova at a ratio of 2 mg KLH to 1 mg peptide and 2 mg Ova to 2 mg peptide using a glutaraldehyde coupling reaction). Mice (three per group) were immunized intraperitoneally with antigen (100 μ g of free peptide or 50 μ g of conjugate) in Freund's complete adjuvant. Three booster injections with the same amount of antigen in Freund's incomplete adjuvant were given at 15, 30 and 45 days after the initial injection. Serum was collected 4 days after the final booster immunization.

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Abbreviations: HAV, hepatitis A virus; MAP, multiple antigen peptide; mAb, monoclonal antibody; KLH, keyhole limpet haemocyanin; Ova, ovalbumin; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.

2.8. Antipeptide antibody assays

Binding of antipeptide antisera to HAV were determined by solid-phase ELISAs. Microtiter plates were coated overnight with human anti-HAV serum at a 1:20000 dilution. Wells were blocked with 1% 3SA prior to the addition of the HAV preparation. After overnight incubation and washing, serial 2-fold dilutions of mouse antisera were added. Anti-species antibodies were commercial horseradish peroxidase conjugates (Sigma).

2.9. Peptide blocking ELISA

Microtiter plates were coated with HAV or synthetic peptides and the anti-VP2-(69–99) serum (1:200) preliminary incubated with the competing peptide (10 µg/ml in 0.1 M PBS) was added.

3. Results and discussion

Peptides were derived as probable B-epitopes based on computer analysis methods of HAV proteins sequences as described elsewhere [8]. In addition we synthesized multiple antigen peptides based on 64–80 and 66–80 segments of VP3. It was reported that anti-peptide antibodies raised after immunisation with the peptide VP3-(62–75) conjugated with KLH were able to bind native HAV [9]. All synthetic peptides and MAPs (Table 1) were tested for binding to anti-HAV antibodies in solid-phase ELISA. We did not detect binding of any peptides 1–17, 2–33, 6–17, 11–25 and 10–33 from VP1 overlapping the reported B-epitope 11–25 from VP1 [3] neither to polyclonal nor to monoclonal anti-HAV antibodies. Peptide VP3-(62–75)

was also not reactive. MAP based on VP3-(66–80) interacted with mAb 1.2.2.2 and slightly less with the IgG fraction. Lengthening of the monomer in MAP-(64–80) by two residues dramatically increased the reactivity of the last with all antibodies.

Peptides VP3-(45–57), VP3-(137–150) and VP2-(69–99) bound only to mAb 1.2.2.2. Peptides corresponding to the N-terminal part of the 107–139 region of VP1 (107–126, 112–126 and 115–126) did not react with any anti-HAV antibodies, whereas those corresponding to the C-terminal part of this region (117–139 and 126–139) bound to the all three mAbs. Peptide VP1-(115–139) which is two residues shorter than VP1-(117–139) interacted only with mAb 1.2.2.2. It is not inconceivable that peptide elongation causes the change of its conformation and in consequence its antigenic properties. The same conclusions can be drawn regarding peptides 69–99 and 80–99 of VP2.

It can be seen from Table 2 that peptides VP1-(126–139) and VP2-(80–99) having non-related structures bound to all antibodies. Furthermore, one can notice that the same antibodies reacted with the peptides of variable composition and were derived from different proteins. At present we have no plausible explanation for these facts, but a related phenomenon was described earlier [10–12].

MAPs, VP1-(115–139), VP1-(117–139), VP2-(69–99), VP2-(80–99) binding to anti-HAV antibodies, VP1-(10–33) and

Table 1
Synthetic peptides from the HAV capsid proteins

| Protein | Segment | Amino acid sequence |
|-------------|---------|--|
| VP1 | 1–17 | VGDDSGGFSTTVSTEQN |
| | 2–33 | GDDSGGFSTTVSTEQNVDPQVGITTXRDLKG |
| | 6–17 | GGFSTTVSTEQN |
| | 10–33 | TTVSTEQNVDPQVGITTXRDLKG |
| | 11–25 | TVSTEQNVDPQVGI |
| | 64–75 | KVPETFPPELKPG |
| | 75–85 | GESRHTSDHXS |
| | 75–92 | GESRHTSDHMSIYKFXGR |
| | 107–126 | YTFPITLSSTSNPPHGLPST |
| | 112–126 | TLSSSTSNPPHGLPST |
| | 115–126 | STSNPPHGLPST |
| | 115–139 | STSNPPHGLPSTLRWFFNLFQLYRG |
| | 117–139 | SNPPHGLPSTLRWFFNLFQLYRG |
| | 126–139 | TLRWFNLFQLYRG |
| | 180–195 | DYKTALGAVRFNTRRT |
| | 209–221 | YAVSGALDGLGDK |
| | 276–298 | XSRIAAGDLESSVDDPRSEEDRR |
| | 288–298 | VDDPRSEEDRR |
| | 290–299 | DPRSEEDRRF |
| VP2 | 42–62 | PLKTSVDKPGSKKTQGEKFFL |
| | 65–85 | SADWLTTALFHEVAKLDVVK |
| | 69–99 | LTTHALFHEVAKLDVVKLLYNEQFAVQGLLR |
| | 73–85 | ALFHEVAKLDVVK |
| | 80–99 | KLDVVKLLYNEQFAVQGLLR |
| | 178–190 | PVWELTIRVWSEL |
| VP3 | 45–57 | GKITHFTTWTISI |
| | 62–75 | AQFPFNASDSVGQQ |
| | 137–150 | PGNELIDVTGITLK |
| | 235–246 | APLYHAXDVTITQ |
| VP4 | 1–23 | MNMSKQGIQTVGSGLDHILSLA |
| MAP-(64–80) | | (FPFNASDSVGQIKVIP) ₈ -K ₄ -K ₂ -K-G |
| MAP-(66–80) | | (FNASDSVGQIKVIP) ₈ -K ₄ -K ₂ -K-G |

ζ = Nle

VP3-(62–75) overlapping already described B-epitopes were chosen for the production of anti-peptide antisera. Anti-peptide antibodies were obtained after immunization with the free peptides VP-1-(115–139), VP-1-(117–139), VP-2-(69–99) and VP-4-(1–23) and their conjugates with ovalbumine. Mouse strains for immunization with free peptides were chosen based on antigen-dependent proliferation of lymph node T-cells [13]. Peptide conjugates were injected into BALB/c mice.

The presence of anti-peptide antibodies in animal sera was tested by ELISA. Pooled sera from three mice immunized with appropriate antigen were used. Of the free peptides only VP-1-(115–139), VP-1-(117–139), VP-2-(69–99) and VP-4-(1–23) induced anti-peptide antibodies (Table 3).

Peptide VP2-(69–99) contains two lysine residues at positions 80 and 85 which can react with glutaraldehyde during the conjugation reaction. We did not obtain anti-peptide antibodies after immunization with the conjugate VP2-(69–99)–Ova. It can be assumed that Lys⁸⁰ and Lys⁸⁵ participate in forming of the antigenic determinant localized on the N-terminal part of VP2-(69–99). Peptides VP-1-(115–139) and VP-1-(117–139) conjugated to ovalbumine also raised anti-peptide antibodies.

Free peptides VP1-(10–33) and VP3-(62–75) did not induce anti-peptide antibodies, while immunization with the peptides conjugated to KLH resulted in peptide binding antibodies production. These data, combined with the results of T-cell epitopes determination [13], indicate the low immunogenicity of VP1-(10–33) and VP3-(62–75). High titre antisera were obtained after immunization with both MAPs. However, antisera to MAP-(64–80) and MAP-(66–80) reacted weakly with the peptide VP3-(62–75) (data not shown). Obviously a new antigenic determinant is formed in MAP constructs to which the main antibody response is directed. This B-epitope is localized close to the C-terminal part of peptides and is shared by both MAPs, since antisera obtained after immunization with the one MAP bound to the homologues antigen and to other MAP with about the same titres (data not shown).

Anti-peptide antisera were also tested in ELISA for their ability to react with native HAV. Sera from non-immunized mice and mice immunized with KLH and Ova served as controls. Only antiserum to free VP2-(69–99) was found to react with native HAV (Table 3). At the same time antisera to VP2-

Table 3
Immunogenicity of synthetic antigens*

| Antigen | Mouse strain | H-2 | Anti-peptide sera | Binding of anti-peptide sera to native HAV |
|-------------------|---------------------------------|-----|-------------------|--|
| VP1-(10–33)–KLH | BALB/c | d | 3 | < 1 |
| VP1-(115–139) | C ₅₇ Bl ₆ | b | 3.5 | < 1 |
| VP1-(115–139)–Ova | BALB/c | d | 3.2 | < 1 |
| VP1-(117–139) | C ₅₇ Bl ₆ | b | 2.5 | < 1 |
| VP1-(117–139)–Ova | BALB/c | d | 3.2 | < 1 |
| VP2-(69–99) | BALB/c | d | 4.2 | 3.2 |
| VP2-(80–99)–KLH | BALB/c | d | 3 | < 1 |
| VP3-(62–75)–KLH | BALB/c | d | 3 | < 1 |
| VP4-(1–23) | C ₅₇ Bl ₆ | b | 4 | < 1 |
| MAP-(64–80) | BALB/c | d | 4.8 | < 1 |
| MAP-(66–80) | BALB/c | d | 5.1 | < 1 |

*Titre of antibodies on the Ig scale.

(69–99) did not block the binding IgG of pooled reconvalescent sera to virus (data not shown). It follows that the B-epitope mimicked by VP2-(69–99) is not immunodominant or in the process of disease development antibodies are formed directed to epitope(s) differing from those represented by VP2-(69–99). It is a distinct possibility that this peptide represents the B-epitope recognized by IgM antibodies that are in patient sera on the initial step of the disease. The result obtained is in agreement with published data [14].

VP2-(69–99) and VP2-(80–99) blocked the binding anti-VP2-(69–99) serum both to the source peptide and HAV (data not shown). Thus, the interaction between antigens and peptide-specific antibodies is highly specific. Antiserum to VP2-(69–99) reacted with VP2-(80–99) (titres 1/10000), but showed no binding to VP2-(73–85) and VP2-(65–85). These latter also had no effect on binding of the antiserum both to VP2-(69–99) and HAV. Thus, from the results obtained it may be deduced that the B-epitope is located near to C-terminal part of 69–99 fragment of HAV VP2.

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Table 2
Reactivity of synthetic antigens with anti-HAV antibodies in ELISA*

| Antigen | Anti-HAV mAb | | | IgG of pooled reconvalescent sera |
|---------------|--------------|-----------|-----------|-----------------------------------|
| | B.2.6.8 | HB-1 | 1.2.2.2 | |
| VP1-(115–139) | 1.3 (–) | 1.3 (–) | 6.2 (+) | 1.6 (–) |
| VP1-(117–139) | 4.2 (+) | 4.9 (+) | 9.6 (+) | 2.0 (–) |
| VP1-(126–139) | 2.9 (+) | 7.1 (+) | 18.2 (+) | 2.4 (+) |
| VP2-(69–99) | 1.5 (–) | 1.6 (–) | 12.2 (+) | 2.3 (+/–) |
| VP2-(80–99) | 2.6 (+) | 2.9 (+) | 22.3 (+) | 2.4 (+) |
| VP3-(45–57) | 1.2 (–) | 1.0 (–) | 2.2 (+/–) | 1/2 (–) |
| VP3-(137–150) | 1.1 (–) | 1.4 (–) | 3.7 (+) | 1.2 (–) |
| MAP-(64–80) | 12.7 (+) | 9.8 (+) | 32.3 (+) | 17.5 (+) |
| MAP-(66–80) | 2.2 (+/–) | 2.2 (+/–) | 5.7 (+) | 2.8 (+) |

The data are expressed as means of triplicate determinations.

*Values of the S/N ratio are presented (see section 2). Data for peptides with a negative result are not shown.

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