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Identification of hepatitis A virus mimotopes by phage display, antigenicity and immunogenicity.

Osmany G. Larralde [*§], Raiza Martinez[1], Frank Camacho[1], Nevis Amin[1], Alicia Aguilar[1], Arturo Talavera[1], David I. Stott[2], Ela M. Perez*[1]

[1]Dept. Molecular Virology, Research Vicepresidency, Finlay Institute, Ave 27 # 19805 e/ 198 y 202, Cod. 11600, POBox 16017, La Lisa, Havana Cuba.

[2]Division of Immunology, Infection & Inflammation, Glasgow Biomedical Research Centre, University of Glasgow, 120 University Place, Glasgow G12 8TA, Scotland, UK.

[§] Present address: Division of Virology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G11 5JR, UK.

[*]Corresponding authors:

elaperez@finlay.edu.cu Tel: 53 7 271 6911, Fax: 53 7 208 6075

0202767L@student.gla.ac.uk Tel: 441413306269, Fax: 441413372236

Abstract

A phage-displayed peptide approach was used to identify ligands mimicking antigenic determinants of hepatitis A virus (HAV) for the first time. Bacteriophages displaying HAV mimotopes were isolated from a phage-display peptide library by affinity selection on serum antibodies from hepatitis A patients. Selected phage-peptides were screened for reactivity with sera from HAV infected patients and healthy controls. Four cloned peptides with different sequences were identified as mimotopes of HAV; three of them showed similarity in their amino acid sequences with at least one of the VP3 and VP1 antigenic proteins of HAV. One clone was recognised by 92% of the positive sera. The phagotopes competed effectively with HAV for absorption of anti-HAV specific antibodies in human sera, as determined by ELISA. The four phage clones induced neutralising anti-HAV antibodies in immunised mice. These results demonstrate the potential of this method to elucidate the disease related epitopes of HAV and to use these mimotopes in diagnostic applications or in the development of a mimotope-based hepatitis A vaccine without the necessity of manipulation of the virus.

Key words: phage display, HAV, mimotopes, vaccine, diagnostic

1. Introduction

Hepatitis A is an acute disease that has considerable morbidity and imposes a large economic burden, especially in developing countries. The etiological agent is *Hepatitis A virus* (HAV), classified in the genus *Hepatovirus* within the *Picornaviridae* family (Cuthbert, 2001).

The IgG antibody response to HAV is delayed compared with IgM and IgA responses but frequently persists for life, providing protection against reinfection. Pre and post-exposure passive immunisation with human immune serum globulin is c.90% effective in preventing hepatitis A, so low levels of neutralizing antibody can give effective protection (Stapleton, 1995).

The P1 region of HAV encodes the three major proteins of the viral capsid: VP1, VP2 and VP3. A fourth viral capsid protein (VP4), essential for virion formation, is not detected in mature viral particles. Each of the capsid proteins is cleaved from the precursor polyprotein by the viral protease 3C (Hollinger and Emerson, 2001). Human HAV strains are classified into a single serotype and four genotypes. Between each of these genotypes, the nucleotide sequence varies at 15-25% of base positions in the P1 region. Despite this, there is good evidence that all human strains of HAV are closely related antigenically. Therefore, infection with any human strain is very likely to provide protection against all relevant human HAV strains (Lemon et al., 1992).

Technical difficulties, such as the slow replication cycle and low yields of virus have hampered for years the detailed characterisation of the antigenic structure of HAV. Most of the information on the antigenic structure of HAV was obtained after the isolation of neutralization-escape mutants because the major neutralization epitopes of HAV appear to be discontinuous (Stapleton and Lemon, 1987). There is a

conformation-dependent immunodominant neutralization site, composed of clustered epitopes, which involves residues 70 and 74 of VP3 and residues 102, 171 and 176 of VP1. Similarly, there is another apparently distinct antigenic site represented by mutants at residue 221 of VP1 (Ping and Lemon, 1992). An additional and still undefined third antigenic site also exists because a monoclonal antibody was produced that effectively neutralises all escape mutants so far isolated (Lemon and Robertson, 1993). Three epitopes within the main VP3-VP1 site are present in 14S pentamers, while two additional epitopes are formed upon assembly of 14S subviral particles in the capsid (Stapleton et al., 1993).

The availability of combinatorial peptide libraries has provided a powerful tool for selecting sequences that mimic conformational epitopes (mimotopes) either structurally and/or immunologically. These mimotopes can be particularly useful in a number of situations, including: the development of vaccines against tumors, infectious diseases or allergic conditions; the design of molecules that act as agonists or antagonists of various biologically-important molecules; and for the development of diagnostic assays (Partidos and Steward, 2002).

The strategy to identify peptides that mimic disease-specific epitopes from phage-displayed random peptide libraries using human sera was previously established for the identification of mimotopes of hepatitis B virus (Folgori et al., 1994) and hepatitis C virus (Prezzi et al., 1996). In the case of HAV, the phage-display technique has only been used to clone monoclonal antibodies from phage-displayed antibody libraries (Wan et al., 1998 and Scholfield et al., 2002).

In this study, a phage-displayed peptide approach was used for the first time to identify ligands capable of mimicking antigenic determinants of hepatitis A virus that

could be useful in the development of a diagnostic kit or a potential antigen for vaccine production.

2. Materials and methods

Sera and anti-HAV assay.

All the serum specimens used in this study were collected with written consent from the donors. Both groups in the study were adults from Havana City, Cuba. All the sera were tested by ELISA and did not have antibodies to HBsAg, HCV or HIV.

Group A: Test serum specimens (n=24) were collected from outbreaks of hepatitis A. Criteria for the diagnosis of acute hepatitis A included a clinical illness compatible with acute viral hepatitis, peak alanine aminotransferase (ALT) values at least 10 times normal, positive for total anti-HAV antibody (ELISA ETI-AB-HAVK-3, DiaSorin Ltd, Italy), positive IgM anti-HAV antibody (ELISA ETI-HA-IgM-2, DiaSorin Ltd, Italy).

All the serum specimens from group A were positive for IgG anti-HAV using an in-house anti IgG ELISA. Briefly, this was performed in multi-well plates (Nunc Maxisorp F8, Life Technologies Limited, Paisley, UK), coated with 100 µl of a solution 10 µg/ml of HAV antigen (Mediagnost, Germany) in 50 mM $NaHCO_3$, pH 9.6 overnight at 4°C, then washed 3 times with PBS/0.05% Tween 20 v/v and blocked with PBS-T/3% BSA w/v for 1 h at 37ºC. 1/100 dilutions of serum samples were added (100 µl/well) in duplicate and incubated 2 h at 37ºC, plates were washed 3 times with PBS-T, then incubated for 1 h at 37ºC with 100 µl/well of goat anti-human IgG/HRP diluted 1:5000 (Sigma - Aldrich, UK), washed and developed for 15 min. with TMB substrate (KPL, EU). The reaction was terminated with 0.1M H_2SO_4 and the absorbance at 450

nm recorded by an automated ELISA reader (Dinex Technologies, UK).

Quantitation of anti-HAV antibody (IU/ml) from human sera was also performed using a quantitative Enzyme-Linked Fluorescent Assay (ELFA, VIDAS Anti-HAV Total, bioMerieux SA, France). One serum sample (AV1) collected from a donor immunised with HAVRIX hepatitis A vaccine (GSK, UK), was also included in this group. The anti-HAV antibodies detected in sera A16, A21 and A53 were of IgG class only and the remaining sera contained antibodies of both IgG and IgM class.

Group B: Test serum specimens (n=23) were collected from healthy donors who had no previous history of viral hepatitis, nor any previous history of administration of immune globulin or hepatitis A vaccine. All serum specimens from this group were negative for total anti-HAV antibodies (ELISA ETI-AB-HAVK-3, DiaSorin Ltd, Italy) .

All serum samples were stored at -20^{0}C until use.

2.2. J404 nonapeptide phage-display library

The J404 bacteriophage display peptide library (PDPL) used in this study was kindly donated by Dr. Jim Burritt (Montana State University, USA, Burrit et al., 1996)

2.3. Affinity Selection.

The methodology followed to identify HAV mimotopes using polyclonal sera is essentially similar to that described by Folgori et al. (1994), with some variations. 100 µl of magnetic microbeads coupled to mouse anti-human IgG (MACS, Miltenyi Biotec, UK) were blocked overnight at 4°C in PBS/0.1% BSA w/v with gentle mixing. After

blocking, the magnetic microbeads were incubated overnight at 4°C with 30 µl of the selecting positive anti HAV serum A08 (610 IU/ml), bound to a magnetic column (Magnetic cell separator MiniMACS, Miltenyi Biotec, UK) and washed 4 times with PBS/0.1% BSA w/v /0.1% Tween 20 v/v (PBSB-T). The microbeads were released from the column and blocked with excess of UV-killed wild type phage M13K07 particles for 4h at 4°C. They were then incubated with 10^{12} colony forming units (CFU) of J404 PDPL overnight at 4°C with gentle mixing, applied to the magnetic column again and washed 6 times with PBSB-T. After washing, bound phages were eluted with 0.1 M glycine HCl, pH 2.2 and the eluate neutralised with 1M Tris HCl pH 9.1. A few microliters of the eluted phage were removed for titering, and the remaining phage amplified in *Escherichia coli* strain TG1 cells [K12, Δ (*lac-pro*), *supE, thi, hsdD5*/F' *traD*36, *proA*$^+$ B$^+$, *lacI*^{q}, *lacZ*ΔM15, Hoogenboom et al., 1991] (Sambrook, J. and Russell, D. W, 2001).

2.4. Immunoscreening in situ.

Plaque lifts of phage colonies were prepared by placing nitrocellulose membrane discs on overlay plates containing 100 colonies approximately, for 4 h at room temperature (RT). The membranes were blocked with PBS/0.1% NP40 v/v/5% non-fat dry milk w/v (PBSNM) for 2h at RT changing the buffer 4 times. A second positive serum, A05 (242 IU/ml, 1/50 dilution in PBSNM) was pre-adsorbed with TG1 *Escherichia coli* extract and UV-killed M13K07 phage for 2h at RT as described (Felici et al., 1996). The pre-adsorbed serum was added to the nitrocellulose discs and incubated overnight at 4°C with gentle mixing, followed by 10 washes with PBS/0.1% NP40 v/v. The washed membranes were incubated with alkaline phosphatase

conjugated goat anti-human IgG (Sigma - Aldrich, UK, diluted 1:5000 in PBSNM) for 4 h at 4°C washed and developed in NBT/BCIP (Pierce, UK) chromogen for 2-5 min.

2.5. Immunoscreening by Slot-Blot.

Positives clones identified by immunoscreening were picked using a Pasteur pipette, transferred to tubes containing 1 ml of LB broth following by an overnight incubation at 37°C and centrifuged at 6000 g, 10 min at 4°C. The supernatant was then transferred to a new tube, incubated for 20 min at 70°C and centrifuged at 6000 g, 30 min at 4°C. Supernatants from phage clones and M13K07 (negative control) were titered and their concentration adjusted to $7.5x10^{7}$ CFU/ml. 50 µl of each sample was applied to a nitrocellulose membrane in a Slot-Blot apparatus. The membranes were blocked with PBSNM for 2h at RT changing the buffer 4 times. Subsequent steps were as described above for immunoscreening in situ and all the clones were probed with three positive (A01, A02, A52) and three negative (B04, B94, B95) anti-HAV sera.

2.6. Phage-ELISA.

In this experiment the test sera from Group A (24 sera) and Group B (23 sera) were used individually in order to evaluate the specific reactivity of the phagotopes. Prior to ELISA each phage clone considered positive in the Slot–Blot was amplified and purified by PEG 8000/NaCl precipitation (Sambrook and Rusell, 2001). Phage clones and M13K07 phage (wild type control) were titred and their concentration adjusted to $2.5x10^{12}$ CFU/ml. Multi-well plates (Nunc Maxisorp F8, Life Technologies Limited, Paisley, UK) were coated with 100 µl of anti-M13 monoclonal antibody (Amersham

Pharmacia Biotech, UK) (10 μg/ml in 50 mM $NaHCO_3$ pH 9.6). Plates were incubated overnight at 4°C then washed 3 times with PBS/0.05% Tween 20 v/v (PBS-T) and blocked with PBS-T/5% non-fat dry milk w/v for 1 h at 37°C. Phage clones and wild type phage (as controls) were added (100 μl/well) and incubated for 4h at RT. Plates were washed three times with PBS - T and test serum added (1/100, pre-adsorbed with TG1 *Escherichia coli* extract and UV-killed M13K07 phage, for 4h at RT). Plates were washed 4 times with PBS-T, incubated for 4 h at 37°C with 100 μl/well of goat anti-human IgG/alkaline phosphatase conjugated (Sigma - Aldrich, UK) diluted 1:5000, washed and developed with p-nitrophenyl phosphate substrate. The absorbance at 405 nm was recorded by an automated ELISA reader (Dinex Technologies, UK). For each serum, average results from two independent experiments were evaluated. Values were considered as positive when the ratio (P/N) of absorbance of phage clones over absorbance of phage M13K07 (wild type control) was > 2.1.

2.7. Sequencing.

An appropriate dilution of phage was plated in LB plates containing kanamycin (Sigma-Aldrich, UK) at 75 μg/ml. Single colonies were excised and phage midipreps (QIA prep Spin, Miniprep KIT, USA) prepared in LB broth containing kanamycin at the same concentration. DNA from phages was sequenced using a geneIII-specific primer, which anneals ~50 nt from the 27-mer insert as described (Burrit et al., 1995). The phage-displayed peptide sequences were aligned using the BLAST algorithm (Altschul et al, 1990).

2.8. Competitive Inhibition assay of HAV mimotopes.

The anti-HAV antibody concentration of positive and negative sera was measured before and after adsorption with each of the four phagotopes and the wild type phage M13K07 using the semiquantitative competitive kit ETI-AB-HAVK-3 (DiaSorin Kit, Italy). The assay was performed according to the supplier's instructions. Four positive and three negative sera were assayed with and without pre-incubation with phages. Briefly, phage clones were incubated with the sera (1:1, 10^8 CFU in 50 μl PBS + 50 μl of serum) for 2 hours at 37ºC, before the measurement of antibody titers. Free HAV was detected with anti-HAV / HRP conjugate. The absorbance at 450 nm was recorded by an automated ELISA reader (Dinex Technologies, UK). The samples were evaluated in triplicate and the experiment was performed twice. The anti-HAV concentration (mIU/ml) before and after incubation with the phage clones was calculated from the calibration curve of the positive control of the kit following the suppliers instructions (five calibrators were prepared by serially diluting the positive control serum 1: 2 v/v, from 80 to 5 mIU/ml). The statistical regression of this curve was 98%. The formula used for calculating the percentage inhibition of anti-HAV by the phage clones is:

$$\% \text{ inhibition} = 100 - (100 \times [ABS] / [NABS])$$

where [ABS] is the antibody concentration after absorption of the serum with phage and [NABS] is the antibody concentration before absorption, calculated from the regression curve of the calibrator of the kit.

2.9. Immunisation of mice.

Phages (BA1-54, BA1-56, BA1-53, BA1-46 and wild type phage M13) were

prepared for immunisation as described by de la Cruz et al., 1988. Phage clones were resuspended in PBS at a concentration of 10^{12} CFU/ml and injected intraperitoneally (0.1 ml/mouse) into BALB/c mice (5 per group) as a 1:1 emulsion with complete Freund´s adjuvant (CFA) for the first immunisation and with incomplete Freund´s adjuvant (IFA) for booster injections, at 0, 14 and 28 days. Animals were bled before each immunisation and at 42 days; sera were collected and stored at -20ºC until evaluation.

2.10. Evaluation of the anti-HAV response of immunised mice.

The specific reactivity against HAV of the serum samples obtained from immunised mice at 0 and 42 days was determined by an indirect ELISA, performed in multi-well plates (Nunc Maxisorp F8, Life Technologies Limited, Paisley) coated with 100 µl of HAV antigen (Mediagnost, Germany, 10 µg/ml) in 50 mM $NaHCO_3$, pH 9.6 overnight at 4°C, then washed 3 times with PBS/0.05% Tween 20 v/v and blocked with PBS-T/3% BSA w/v for 1 h at 37ºC. 1/80 dilutions of serum samples were added (100 µl/well) in duplicate and incubated 2 h at 37ºC. Plates were washed 3 times with PBS-T, then incubated for 1 h at 37ºC with 100 µl/well of goat anti-mouse IgG/HRP (Sigma - Aldrich, UK, diluted 1:5000), washed and developed for 15 min. with TMB substrate (KPL, EU). The reaction was terminated with 0.1M H_2SO_4 and the absorbance at 450 nm recorded by an automated ELISA reader (Dynex Technologies, UK). The optical density ratio of immune serum (day 42/day 0) was determined for each mouse individually. The data of each group were analyzed by the Kruskall -Wallis Test and Box and Whisker Plot, using the program Statgraphics Plus 5.0.

2.11. Cell culture.

FRhK-4 cell line (ATCC CRL 1688, USA) was propagated in DMEM (GIBCO, USA) plus 10% fetal calf serum v/v (FCS) (GIBCO, USA) at 37 ºC with 5% CO_2.

2.12. Virus titration.

HM175/18f cytopathic clone of HAV (ATCC VR-1402, USA) was grown in FRhK-4 cells and the titer determined in 96 well plates (TC Microwell 96F Nunc, Fisher Scientific, UK) with confluent monolayers of FRhK-4 cells. Eight replicate wells were inoculated with 50 µl of HAV (ten fold dilutions) in DMEM (GIBCO, USA). After one hour of viral absorption at 37ºC, 150 µl of DMEM/2% FCS v/v were added and incubated for seven days at 37ºC in a 5% CO_2 atmosphere. The cytopathic effect (CPE) was assessed by microscopy and viral titer calculated by the method of Reed and Muench, 1938.

2.13. Neutralization of HAV.

Two fold dilutions of hyperimmune sera (collected at 42 days), pre-immune sera (negative control) and a neutralizing monoclonal antibody against HAV (positive control Mab 7E7, Mediagnost, Germany, 25 µg/ml final concentration), were incubated with a fixed dilution of HAV (10^3 $TCID_{50}$/ml). Five replicates of each sample were inoculated in 96 well plates, seeded with FRhK-4 cells, and incubated for 1h at 37ºC. At the end of this period the monolayers were washed three times with PBS and DMEM/2% FCS v/v added. Plates were incubated for seven days at 37ºC in a 5% CO_2

atmosphere and the cytopathic effect (CPE) was observed daily. The neutralization titres of the sera were scored as the reciprocal of the last dilution of serum that inhibited virus growth in more than 50% of the inoculated cell monolayers.

3. Results

3.1. Selection of HAV mimotopes.

A bacteriophage display peptide library (PDPL) derived from M13mp18 vector was chosen as a source of epitopes. This is a randomised linear peptide library that contains $5x10^8$ unique phage, each expressing a different nonapeptide sequence fused to the amino terminus of the pIII capsid protein. The J404 PDPL offers the benefits of high copy replicative form, large plaque formation, and a kanamycin resistance gene allows selection of colonies of *E.coli* infected by the phage (Burrit et al., 1996).

In this study the sera were classified as group A or B according to the presence or absence of anti-hepatitis A antibody respectively (see Materials and Methods).

Serum A08 from a convalescent hepatitis A patient with 610 IU/ml of anti-HAV antibody was chosen for affinity selection of the library. 10^{12} CFU/ml of J404 PDPL were affinity selected and $2.4x10^3$ CFU/ml of bound phage were eluted. The first round of affinity selection was amplified to $1.5x10^{12}$ CFU/ml, plated and subjected to immunological screening in situ using A05 serum from a convalescent hepatitis A patient. 75 positive phage clones were identified (50% of the colonies plated were positive). When sera are used as selectors, multiple selection steps do not necessarily favour enrichment of disease-specific versus non-specific phagotopes, as non-disease-specific antibodies that would select irrelevant phagotopes may also be present in the serum (Felici et al., 1996). Hence, it is advisable to reduce the number of selection

rounds to a minimum (in most cases one round is enough), and probe a large number of selected clones by immunoscreening to identify the positives ones (Folgori et al., 1998). Since there was no enrichment after a second round of affinity selection (48% of colonies positive), the 75 selected clones from the first round of panning, were tested. Three positive sera (A01, A02 and A52) and three negative sera (B04, B94 and B95) were used for pre-screening by Slot Blot analysis of the BA1 phage clones.

Several clones from the first round (BA1) were recognised by anti-HAV positive sera and not by negative sera. Immunological screening by Slot-Blot is summarized in Table 1. Seven clones were identified from the seventy five selected by colony immunoscreening which were recognised by at least two of the positive sera and none of the three negative sera. Clones that gave positive reactions with at least one negative control serum were considered non-disease-related.

3.2 Characterisation of HAV mimotopes using sera from convalescent hepatitis A patients.

The final step of the procedure for selecting disease-specific phagotopes involves testing isolated clones for their reactivity with a large panel of positive and negative sera. This analysis was carried out by phage-ELISA, in which purified phage clones are tethered by an anti-phage monoclonal antibody (MAb) onto the surface of a multiwell plate. The Mab, which specifically recognizes the major capsid protein (pVIII), links the phage to the plate, leaving peptides displayed as a fusion with the pIII capsid protein free to interact with serum antibodies.

Clones BA1-46, BA1-53, BA1-54 and BA1-56 did not react with any of the 23 sera from a control population of healthy individuals (group B), thus confirming that the

selected phagotopes are disease-specific. Clones BA1-03, BA1-06 and BA1-51 were considered non-disease-related, and discarded from the study as they were recognized by 4.3, 56.5 and 47.8% respectively of the sera from group B (data not shown).

The results shown in Table 2 indicated that 100% of the tested population of convalescent patients from group A contained antibodies able to recognise at least one of the selected phagotopes, phage BA1-56 was recognized by 92% of the tested sera. All four phagotopes were recognized by 28% of the sera, including serum AV1 from a vaccinated individual. The strongest reaction occurred with sera A12 and A16. The sera A01, A02, A05 and A08 were exhausted after the firsts round of selection and slot blot. We used the serum A52 as positive control of the slot blot results. The selected clones reacted with sera containing both IgM and IgG antibodies against HAV.

3.3.Mapping the HAV mimotopes.

The deduced amino acid sequences of the mimotopes shared several amino acids at the same positions as regions of the P1 and P3 proteins of the four human HAV strains: HM-175, CR326, MBB and LA. There was no similarity with the VP4 and VP2 proteins. Comparison of the amino acid sequences of the mimotopes with the P1 region from strain HM-175 (Cohen et al., 1987) is shown in Fig. 1. Mimotope BA1-54 exhibited discontinuous amino acid (aa) sequence similarity with two regions of the VP3 capsid protein (Fig. 1A), viz. 5 residues in the region aa 66 to 80 and 5 residues in region aa 117 to 123. Mimotope BA1-56 also exhibited discontinuous homology with 5 residues of the VP3 protein in region aa 69 to 83, suggesting that BA1-54 and BA1-56 resemble two discontinuous epitopes in the VP3 capsid protein. No similarity could be detected between the VP3 protein and mimotopes BA1-46 and BA1-53. Mimotopes

BA1-53 and BA1-56 exhibited discontinuous homology with two regions of the VP1 capsid protein (Fig. 1B). Mimotope BA1-53 displayed 6 residues homologous with VP1 at positions 300 to 302, 320, 322 and 324. Mimotope BA1-56 also displayed 6 residues homologous with VP1 at positions 14 to 16, 221, 226 and 227. Therefore, mimotopes BA1-53 and BA1-56 resemble two discontinuous epitopes in the VP1 capsid protein. No similarity could be detected between VP1 and mimotope BA1-54. The amino acid sequence of BA1-46 (SHSQLGPPVGPP) did not exhibit similarity with any viral protein and it had a hexameric sequence.

3.4. Mimotopes compete with HAV for binding to antibodies in convalescent human sera.

The reaction of the four mimotopes with anti-HAV antibodies is shown in Fig. 2 as the percent inhibition of binding of anti–HAV serum to HAV compared with the unabsorbed sera. BA1-56, BA1-54 and BA1-53 inhibited the binding of anti-HAV antibodies in all four sera tested , while BA1-46 inhibited three of the anti-HAV sera. Inhibition by wild type phage was negligible. Inhibition of the reaction by all four phagotopes under study was greater that 3σ + inhibition after absorption with wild-type phage, depicted by the bar in Figure 2. No reaction was detected in the negative control sera.

3.5. Immunogenicity of HAV mimotopes.

Groups of mice were immunised with the phage clones to determine whether they have the capacity to induce production of specific antibodies against HAV. All the

immunised animals developed a response against HAV by day 42.

The Kruskall Wallis Test showed a statistically significant difference ($p= 0.002$) amongst the medians of the groups at the 95,0% confidence level. The Box and Whisker Plot analysis (Figure 3) showed, that clones BA1-54 and BA1-46 induced a higher response than BA1-56 and BA1-53; wild type phage induced the lowest response.

3.6. Virus neutralisation.

Hyperimmune sera obtained from mice immunised with the four mimotopes neutralised HAV infectivity at titers of 4 – 16, calculated as the reciprocal of the highest serum dilution capable of reducing HAV (HM175/f8 strain) growth by more than 50%. The positive control anti-HAV Mab (7E7) produced the same effect at 1:40 dilution (Table 3). In contrast, the pre-immune sera and sera from mice immunised with wild type phage had no virus neutralising capacity at any of the dilutions tested.

4. Discussion

Recent work has shown that phage-displayed peptides selected using antibodies raised against pathological antigens can be an important tool for both diagnosis and disease prevention (Delmastro et al., 1997; Enshell-Seiffers et al, 2001; Irving et al., 2001; Zhang et al., 2001; Marston et al., 2002; Beckman et al., 2005). Such phage-displayed peptides (mimotopes) do not necessarily have sequence homology with the antigen, but have sufficient conformational homology to induce high affinity antibodies that bind to both the mimotope and the natural antigen (Collins, 1997).

This approach has previously been used to identify mimotopes of hepatitis B and hepatitis C virus (Folgori et al., 1994; Prezzi et al., 1996) but not HAV, although antibody-phage libraries have been used to obtain monoclonal antibodies against HAV (Wan et al., 1998 and Scholfield et al., 2002). In the work described here, the application of a phage-peptide library for cloning and identifying peptides that resemble the antigenic structure of HAV epitopes has been exploited for the first time.

HAV mimotopes were isolated by a three-step procedure: a) Affinity selection of the random peptide library with a positive serum from group A containing a high titer of anti-HAV antibody; b) Colony immunoscreening of the enriched phage population for epitopes also recognized by a second positive serum containing a high titer of anti-HAV antibodies. c) screening by Slot Blot and ELISA using panels of positive sera (group A) and negative sera (group B). The secondary screening by slot blot with a reduced panel of sera (three positive and three negative sera) facilitates the selection of clones before screening with the complete panels of sera, reducing the laborious work of selection by ELISA of a large number of non-specific clones.

Four peptides that mimic antigenic determinants of HAV with high specificity were identified. Both IgG and IgM isotypes have been shown to neutralize HAV (Lemon and Binn, 1983). The IgG response to HAV is delayed compared with the IgM response but is long-lived and accounts for resistance to reinfection (Cuthbert, 2001). The HAV mimotopes reacted well with both human IgG and IgM anti-HAV antibodies and there was no correlation between anti-HAV immunoglobulin isotype and mimotope.

The concept of a mimotope is based on the assumption that the only essential criterion for effective binding of antibody to a peptide is that complementarity between the antigen-combining site of the antibody and the molecular surface of the binding

peptide is maintained in regard to both shape and charge. It follows, therefore, that any antibody-binding peptide deduced without recourse to sequence information should be defined as a mimotope, although it may not necessarily be an accurate reproduction of the epitope (Mattioli et al., 1995).

Three of the selected mimotopes showed partial sequence homology with at least one of the most antigenic proteins of HAV known so far, viz. VP3 and VP1.

Mimotope BA1-46 was recognised by 56% of the positive sera (Table 2). No sequence homology was found between this mimotope (SHSQLGPPVGPP) and any of the structural proteins of HAV. The sequence of this peptide corresponded to a hexapeptide instead of the expected nonapeptide. For this reason, the sequencing was repeated three times, always with the same result, suggesting that a mutated phage was selected. It had the ability to recognise the antibodies present in convalescent sera and to induce virus neutralising antibodies in mice. The absence of sequence similarity with HAV proteins could be due to it being a functional mimotope rather than a structural one, since a consensus peptide sequence may not resemble any known region of a linear protein if it mimics a discontinuous region that exists only on the surface of a correctly folded protein (Burrit el al., 1995; Nauseef et al., 1995).

Mimotope BA1-54 was also recognised by 56% of the positive sera and showed partial homology with two regions of the VP3 capsid protein. The VP3 (110-121) sequence is highly conserved among HAV strains and was described as a continuous epitope by Bosh et al., (1998). This mimotope showed homology with the FDFXV motif of VP3 (117-121), which is implicated in the epitope structure of the VP3 (110-121) sequence because shorter peptides such as VP3 (110-119), VP3 (110-117) and VP3 (110-116) and a tandem repeat of VP3 (111-116) failed to react with convalescent sera (Bosh et al., 1998). Also, both BA1-54 and VP3 show evidence of a β-pleated

sheet structure in the region equivalent to aa 117-124. On the other hand, the conserved RGD motif (aa 112-114) has been involved directly in the interaction of antibodies with the major antigenic loop of foot-and-mouth-disease virus (Verdager et al., 1995) and is necessary to induce the beta-structure of the peptide in the presence of liposomes (Perez, 1998). Despite some residues of homology with VP3 (110-121), it is not possible to conclude that BA1-54 mimics this linear epitope of the VP3 protein. BA1-54 was also found to have partial homology with the VP3 (66-80) region, especially around Asp^{70}, which is highly conserved among human HAV strains. VP3 Asp^{70} was identified by neutralization escape mutants as playing a critical role in the structure of an immunodominant neutralization site of HAV (Nainan , 1998; Ping et al., 1992) . As no crystallographic data on the structure of HAV exist, it is difficult to predict whether BA1-54 has a structural resemblance with these homology regions of VP3.

Mimotope BA1-53 showed the lowest score of recognition by the positive sera (28%) and has discontinuous similarity with the VP1 (300-324) sequence.

Mimotope BA1-56 was recognized by 92% of the positive sera. This is the only mimotope that displays partial homology with both VP1 and VP3 proteins. It has homology with the SXSV motif of VP3 (69-72), and with the SVT motif of VP1 (Burrit et al., 1996; Folgori et al., 1994). A synthetic peptide VP1 (11-25), including this SVT motif, induced anti-HAV neutralizing antibodies (Emini et al., 1985) but was not useful for immunodiagnosis of acute hepatitis A (Gómara, 2000). Chimaeric poliovirus expressing the VP1 (13-24) sequence induced a weak neutralizing response (Lemon, 1992). BA1-56 also shows partial homology with VP1 (221-227) including Lys^{221}. As in the case of Asp^{70}, this residue was identified by neutralization escape mutants and is located in an independent neutralization site of HAV (Ping and Lemon, 1992). Taking into consideration the presence of IgM antibodies against HAV in most of the sera that

recognise BA1-56, indicating a recent infection and the homology of this mimotope with residues at neutralization sites, it is possible to propose that it mimics important antigenic determinants of HAV and therefore has potential to be used for diagnostic applications.

Since attempts to determine the atomic structure of HAV by x-ray crystallography have been unsuccessful and only medium resolution images were obtained by cryo-electron microscopy (Martin and Lemon, 2006), another element in the structural analysis that could be important is the evaluation of similarities in the physicochemical properties of non-homologous amino acid residues in the linear sequence. As an example, similarities in the size, polarity, net charge, or aliphatic character of residues between BA1-56 and segments 68 – 83 of VP3 and with region 221-231 of VP1 (data not shown) were observed. These physicochemical similarities could explain the recognition of the cloned peptides by anti-HAV antibodies, despite the absence of complete homology with the original protein.

It would be interesting to perform further experiments to elucidate the functional significance of discontinuous similarity of one peptide with the regions of two different viral proteins (BA1-56) or the similarity of one peptide with two regions of the same protein (BA1-54 and BA1-56). Other authors have explained many aspects of cell signalling, trafficking and targeting governed by interactions between globular proteins domains and short peptide segments; these domains often bind multiple peptides that share a common sequence pattern or "linear motif" (Neduva et al, 2005).

Inhibition of the reaction of the human convalescent sera with HAV after absorption with the phage clones supports the hypothesis that the peptides mimic HAV epitopes, blocking the reaction of serum antibodies with the virus. Differences in absorption of the antibodies by the different phage clones can be explained by

differences in the frequency of antibodies against the corresponding epitope and in the affinity of the antibodies for the mimotope. Although BA1-46 has no homology to any viral sequence it was able to displace the virus in three of the four sera evaluated.

If the selected peptides are able to adopt conformations similar to regions of viral proteins, it is predicted that they should induce an antibody response against the virus in vivo (Clark and March, 2004; Perea et al., 2004). Immunisation of mice with the selected phage-mimotopes induced production of anti-HAV antibodies in all the animals by 42 days post immunization, compared with a much lower background response induced by the wild-type phage. Similar results were obtained by other authors using different phagotopes mimicking antigens of HBV and HCV (Prezzi et al., 1996; Delmastro, 1997).

Comparisons of available neutralisation tests have shown significant differences in the sensitivity of these assays (Lemon, 1993; Lemon et al., 1997). The neutralisation assay used here is similar to the HAV cytopathic inhibition assay (Beales et al., 1996), which is less labour intensive and simpler than RIFIT (Radio Immunofocus Inhibition Test). Antibodies induced in response to the mimotopes not only bound specifically to HAV, but also neutralised its infectivity and this correlated positively with their anti-HAV response measured by ELISA. Differences between the neutralisation titres of sera induced by different mimotopes could be due to differences in immunogenicity or in the affinity of the antibodies for the viral epitopes. The higher neutralization titer of Mab 7E7 compared with the mouse hyperimmune sera is probably due to its higher concentration and purity. Interestingly, the BA1- 56 sequence has three residues in the same region (14-16) as a synthetic peptide of HAV VP1 (11-25) that induced low neutralizing titers (1/10) measured by RIFIT (Emini et al., 1985)

5. Conclusions

The usefulness of phage display libraries for the isolation and identification of peptides that mimic biologically relevant viral epitopes, as defined by their recognition by antibodies against the virus has been demonstrated in this paper. The ability of the four mimotopes obtained to bind specifically to serum antibodies from convalescent patients could be used to develop a diagnostic assay for hepatitis A. Their capacity to induce neutralising antibodies against HAV in mice suggests that these mimotopes have the potential to produce a mimotope-based vaccine against hepatitis A virus, without requiring growth and manipulation of the virus, with its inherent high cost and biohazard risks.

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Figure legends

Fig 1**.** Deduced amino acid sequences of the mimotopes and alignment with the P1 region of HAV strain HM-175.

(A) Alignment of mimotopes BA1-54 and BA1-56 with VP3 capsid protein.

(B) Alignment of mimotopes BA1-53 and BA1-56 with VP1 capsid protein. Mimotope names are given on the left of their relative amino acid sequence. Partial sequences of each HAV capsid protein are shown underneath the mimotopes for comparison. Numbers refer to amino acid positions in the original capsid proteins after cleavage by protease 3C. Letters in bold represent residues shared between the mimotope and the HAV protein. Arrows show sites of neutralization-escape mutations: VP3 Asp^{70} and VP1 Lys^{221} (33, 34).

Fig. 2. Inhibition of human anti-HAV antibodies by the four selected mimotopes. The antibody concentration before and after absorption with the phages was calculated from the regression curve of the calibrator. The inhibition of binding of anti-HAV antibodies to HAV was calculated as: % inhibition = 100- (100 x ABS/NABS), where ABS is the concentration of antibodies after absorption with the phages and NABS is the concentration of antibodies before absorption. M13 = wild-type phage M13K07. The bar parallel to the X axis represents the average +3σ of the % inhibition of anti-HAV antibodies after absorption with wild-type phage (21.7 %).

Fig. 3. ELISA of the specific reactivity with HAV of sera from mice immunized with the four phagotopes and phage M13K07 (mice were bled on days 0 and 42, serum dilution used = 1/80). The results are represented in a "Box and Whisker Plot" as the ratio of OD values of serum from each immunized mouse at 42 days / OD values of

serum at day 0. The box encloses the middle 50 percent, the median is represented as a line and the mean is plotted as a point, inside the box. Corresponding medians are 5.2 for BA1-56 clone, 9.6 for BA1-54 clone, 5.5 for BA1-53 clone, 8.9 for BA1-46 clone and 3.1 for M13K07 phage. Note that the wild type phage induced the lowest response, which differed statistically significantly from the phagotopes ($p = 0.002$).

Table(s)

Table 1. Slot-Blot analysis of bound phage clones after first round of selection.

Phage clones	Anti-HAV negative sera			Anti-HAV positive sera		
	B04	B94	B95	A01	A02	A52
BA1-03	+/-	-	-	+	-	3+
BA1-06	-	-	-	+	3+	3+
BA1-46	-	+/-	-	3+	3+	+/-
BA1-51	-	-	-	3+	+	-
BA1-53	-	-	-	+	+	-
BA1-54	-	-	+/-	+	3+	+/-
BA1-56	-	-	-	+	+	+

(+) positive, (3+) strong positive, (+/-) border line, (-) negative

Table 2. ELISA reactivity of sera from convalescent hepatitis A to phagotopes[a]

Sera	BA1-46	BA1-53	BA1-54	BA1-56
A06	1,1	1,4	2,5	1,8
A07	1,3	2,2	1,5	1,9
A11	1,5	1,3	1,6	2,2
A12	3,2	4,5	6,0	9,1
A13	1,3	1,2	1,7	2,3
A16	3,3	5,2	9,6	12,0
A18	2,3	1,9	2,3	3,2
A21	1,2	1,3	1,7	2,3
A22	1,4	1,4	1,9	2,2
A23	3,4	2,4	4,1	5,7
A25	3,0	1,0	2,0	3,0
A26	1,5	1,3	2,7	2,2
A27	3,1	3,5	5,6	5,7
A28	3,2	1,1	1,8	2,2
A29	3,3	4,2	4,3	5,3
A30	3,5	2,5	3,5	3,0
A31	2,7	1,8	2,7	2,7
A32	2,3	1,7	3,2	3,5
A33	4,0	2,0	2,1	3,1
A35	1,5	1,1	1,7	2,2
A52	1,5	1,1	2,0	2,2
A53	1,9	1,3	1,9	2,2
A54	2,4	1,3	1,9	3,7
A55	1,9	2,0	2,8	2,7
AV1	2,8	3,4	4,7	4,5

[a]Shaded cells indicate positive results the of the test serum Group A. Values were considered as positive when the relation P/N > 2.1 in reference of the wild type phage.

Table 3. Neutralisation titre of hiperimmune serum/phage clone

Phage clone	Serum 1	Serum 2	Serum 3	Serum 4	average*
BA1- 46	16	16	16	16	16
BA1- 56	8	8	4	16	9
BA1-53	0	0	8	8	4
BA1-54	4	8	4	8	6

* Reciprocal of the highest serum dilution capable of reducing HM175/f8 strain growth by 50%. The preimmune sera and the antiserum of the wild type phage exhibited no effect. The Mab 7E7 used as a positive control showed an average of 40.

Figure(s)
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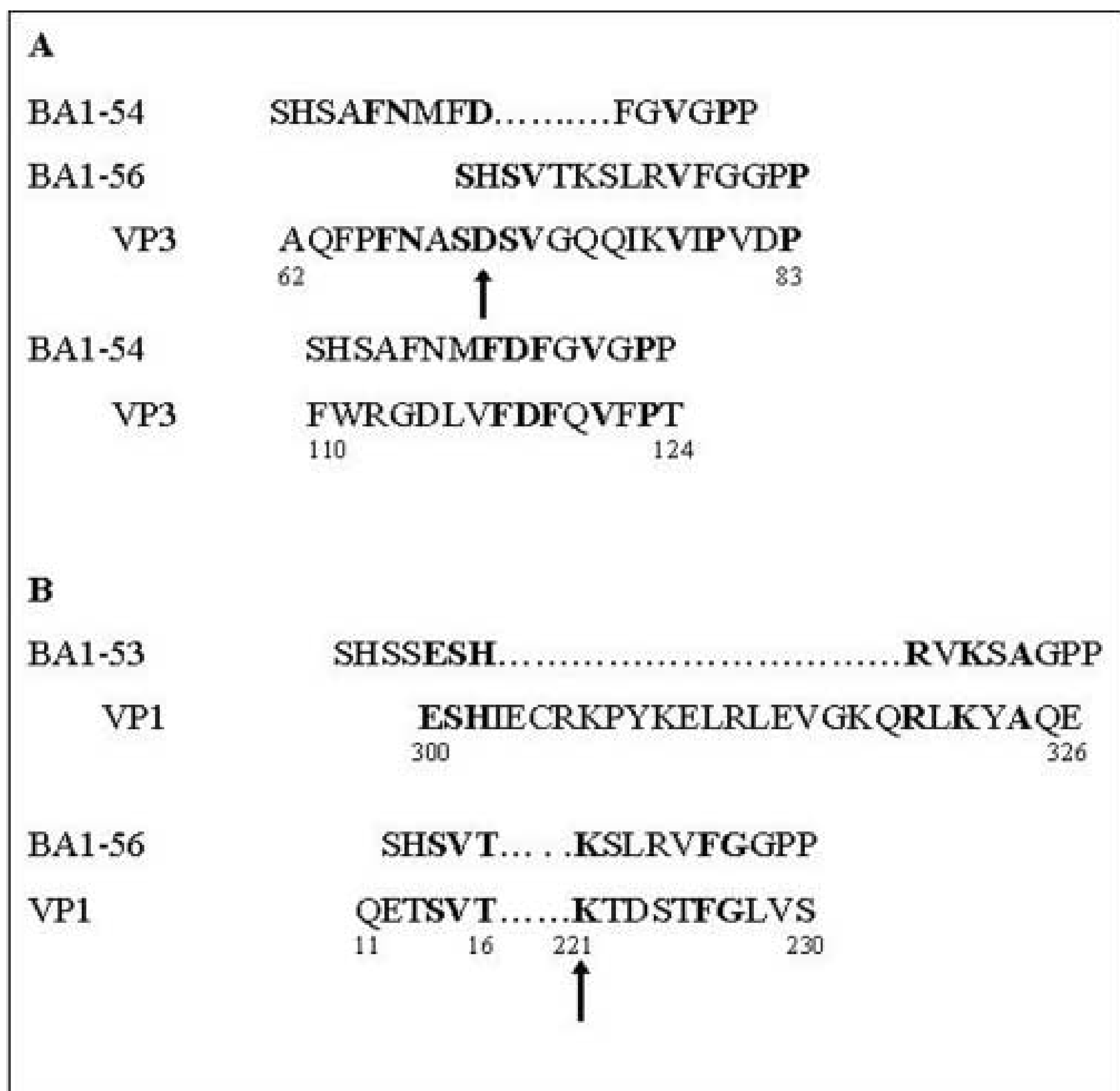

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Figure 2

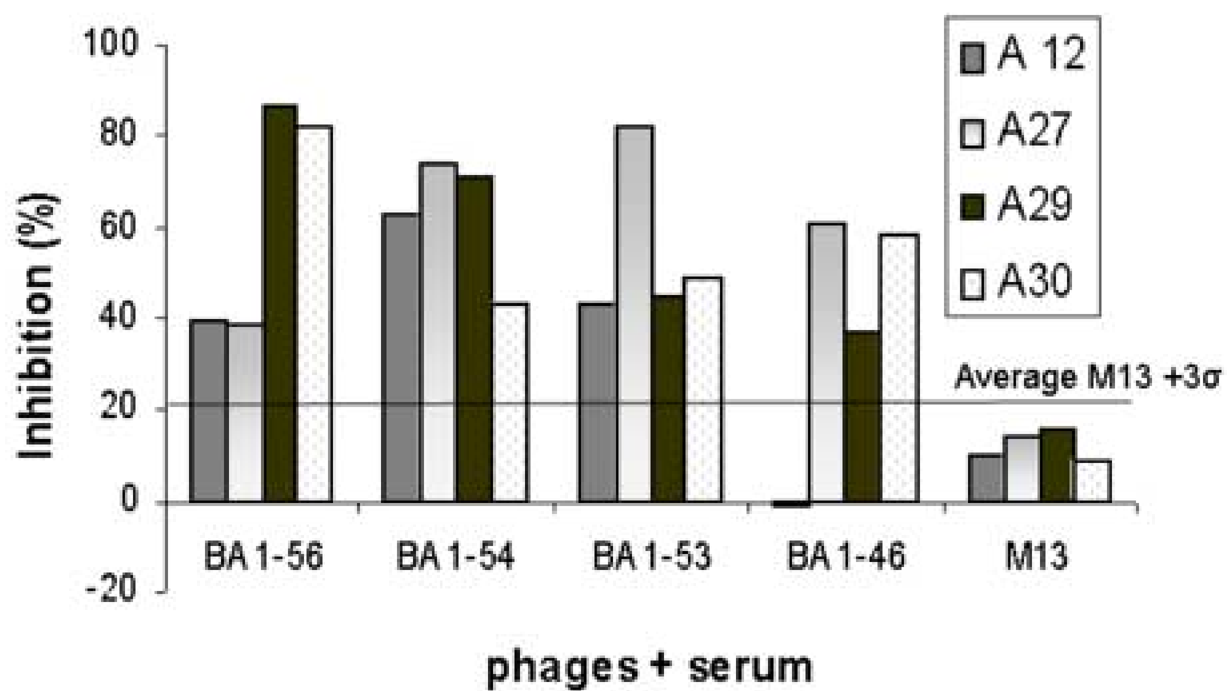

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Figure 3