Peptide mimics selected from immune sera using phage display technology can replace native antigens in the diagnosis of Epstein–Barr virus infection

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There is an expanding area of small molecule discovery, especially in the area of peptide mimetics. Peptide sequences can be used to substitute for the entire native antigen for use in diagnostic assays. Our approach is to select peptides that mimic epitopes of the natural immune response to Epstein–Barr virus (EBV) that may be recognised by antibodies typically produced after infection with EBV. We screened a random peptide library on sera from rabbits immunised with a crude preparation of EBV and serum antibodies from a patient with a high titer of EBV antibodies. We selected four peptides (Eb1–4) with the highest relative binding affinity with immune rabbit sera and a single peptide with high affinity to human serum antibodies. The peptides were coupled to the carrier molecule BSA and the recognition of the peptides by IgM antibodies in clinical samples after infection with EBV was measured. The sensitivities were Eb1 94%, Eb2, 3, 4 88%, H1 81% and all had 100% specificity. This study illustrates that the phage display approach to select epitope mimics can be applied to polyclonal antibodies and peptides that represent several diagnostically important epitopes can be selected simultaneously. This panel of EBV peptides representing a wide coverage of immunodominant epitopes could replace crude antigen preparations currently used for capture in commercial diagnostic tests for EBV.
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Introduction

Epstein–Barr virus (EBV) or human herpesvirus 4 was established as the major cause of infectious mononucleosis commonly known as glandular fever (Epstein and Achong, 1973). The virus has also been linked to more severe diseases and is associated with severe infection in post-transplantation and immunocompromised individuals and some malignancies, including Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease and immunoblastic lymphoma. More recently, EBV infection was linked with autoimmune diseases such as systemic lupus erythematosus (Gross et al., 2005). There is currently a spectrum of diagnostic strategies (immunofluorescence assay, western blot, PCR and ELISA; reviewed by Hess, 2004), but there is no single standardised diagnostic test for EBV infection to date. Therefore, the search for a reliable routine diagnostic test is an important

area for investigation. Current commercial serological diagnostic tests use ELISA to measure IgG and IgM antibodies to viral antigens, including viral capsid antigen (VCA), p18 an immunodominant region of VCA (Chan et al., 1998), EBV nuclear antigens (EBNA) and early antigen (EA-D); usually, a combination of these tests is required to confirm acute infections (Gartner et al., 2003).

Our aim in this study is to produce a panel of peptide mimics, which represent diagnostically important EBV epitopes. Our strategy is to find alternatives to the authentic EBV crude antigen used in current commercial ELISA-based tests. The use of these peptides could provide a more specific and cost-effective commercial diagnostic test. Peptides may also eliminate the high proportion of unwanted epitopes represented in the crude antigen preparation which can often result in false-positive cross-reactions. Cross-reactivity with rheumatoid factor and other herpes viruses has been described in serological EBV diagnostics (Gartner et al., 2003).

A peptide mimotope or epitope mimic is a peptide that will mimic the antibody binding site on the antigen and compete with the native protein for binding. Therefore, peptide mimotopes representing antigenic epitopes that are recognised by serum antibodies produced after infection with EBV may eliminate the need to use the whole antigen in diagnostic assays.

We have previously generated EBV peptide mimotopes by identifying peptides against four different monoclonal antibodies from a phage-displayed random peptide library (Casey et al., 2006; Tschiggerl et al., 2008). These peptides were found to represent different EBV epitopes and were useful for detection of EBV IgM antibodies in clinical samples with 100% specificity and 54–88% sensitivity. Two of the most effective peptides F1 and Gp125 were subsequently conjugated to BSA and used in screening of >200 EBV serum samples which resulted in improved sensitivity (95% and 92%; Casey et al., 2006). A limitation of this approach, however, is that individual mAbs represent only a fraction of the total antibody response to antigen, whereas the use of polyclonal antibodies is likely to increase the chances of selecting useful mimotopes as it samples the entire population of antibodies in the serum. MAbs are not always available for every infectious disease agent necessitating the use of polyclonal reagents in these cases. Furthermore, the antibodies in the polyclonal immune response will react with all the immunodominant epitopes associated with EBV virus infection.

The aim in this study was to select peptide mimics specific for epitopes in polyclonal sera from our phage-displayed peptide library. These peptides may be useful for detecting antibodies reactive with cognate antigen diagnostically. In this current study, we have chosen two different approaches for selection of peptide ligands using polyclonal sera. First,

we isolated the IgG fraction from patients' sera containing a high titer of EBV antibodies. Second, we immunised a rabbit with EBV and affinity-purified antibodies.

In this study, we show that polyclonal antibodies can be used to select peptide ligands from a random peptide library and these epitope mimics are useful for diagnosis, with a specificity and sensitivity similar to those peptide mimics selected against EBV mAbs described in our previous study (Casey *et al.*, 2006). This study is also the first example of screening a random peptide library with polyclonal antibodies from an immunised rabbit and has allowed isolation of peptide mimotopes to several important diagnostic epitopes simultaneously.

Materials and methods

Preparation of polyclonal EBV antisera

A New Zealand white rabbit was immunised intramuscularly with 200 µg EBV-infected cell extract (crude EBV; ABI, MD, USA) emulsified in 0.5 ml Freund's complete adjuvant. Two booster doses diluted 1:1 in Freund's incomplete adjuvant followed by a final double-dose boost were performed in 21 day intervals.

Human serum samples

A panel of 40 individual human serum samples were provided by Queensland Medical Laboratory (Brisbane, Australia). The positive sera ($n = 16$) were collected from individuals with recent or early stage of infectious mononucleosis and were tested for the presence of IgM antibodies to EBV using a commercial diagnostic test (PanBio Ltd). An individual seropositive serum sample with a high titer of IgM and IgG EBV antibodies was selected for purification. The negative sera ($n = 16$) were collected from patients having no previous exposure to EBV infection and were defined as seronegative using the commercial diagnostic test. Putative cross-reactive sera were also screened ($n = 8$), two Parvovirus (Parvo), two Herpes Simplex virus (HSV), two Cytomegalovirus (CMV) and two Rheumatoid factor (RF), to analyse the specificity of binding.

Affinity purification of rabbit and human IgG

The IgG fraction from an EBV-immunised rabbit and human serum with a high titer of antibodies to EBV were purified using Protein G sepharose (2.5 ml column; Pharmacia), using the manufacturer's instructions. Briefly serum was diluted 1:5 in PBS and passed through a 0.2 µm syringe filter prior to being applied to the resin, and antibodies were eluted with 0.1 M glycine pH 3.0, neutralised and dialysed against PBS with three buffer changes.

Phage library and selection

For selection of phage peptides to affinity purified sera from an EBV-infected patient and an EBV-immunised rabbit, we screened our AdLib 1 library (AdAlta Pty Ltd) a linear peptide library of 20 random amino acids displayed as N-terminal fusions to protein III of filamentous phage M13 (Casey *et al.*, 2004, 2006, 2008). A similar panning strategy was used as described in our previous study (Casey *et al.*, 2006). Briefly ELISA wells were coated with 10 µg/ml purified rabbit or human anti-EBV IgG preparations and peptides were selected from the library of $>5 \times 10^8$ random peptides by performing six rounds of panning. The stringency of washing was increased in each subsequent round of panning to enrich for phage peptides that bound specifically to the target antibodies.

Peptide synthesis

Peptides were synthesised to $>70\%$ purity by GL-Biochem (Shanghai, China). Peptides Eb1, Eb3 and Eb4 were dissolved in dimethyl formamide at 1 mg/ml used fresh or stored in aliquots at $-20°C$. Eb2 was soluble in PBS and stored in a similar manner. Gp125 and F1 peptides were prepared as previously described (Casey *et al.*, 2006).

Peptide conjugation to BSA

Peptides were synthesised with four additional glycine residues and an additional cysteine residue at the C′ terminus (Gly$_4$Cys). The glycines provide a small spacer region between the peptide and the additional cysteine residue allows for conjugation to BSA via the heterobifunctional cross-linker succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, Pierce), using the same method as described in our previous study (Casey *et al.*, 2006). Briefly 100 molar excess of SMCC was added to 5 mg BSA (Thermo, New Zealand) in 0.1 M sodium phosphate/0.15 M sodium chloride buffer for 2 h mixing at room temperature. To remove excess linker, the mixture was desalted using a PD-10 column equilibrated with the same buffer containing 0.1 M EDTA. For conjugation, 1 mg of peptide was incubated with 1–2 mg of BSA-SMCC in the presence of 30% DMSO for 2 h. The final BSA-conjugated peptide was desalted using a PD-10 column into PBS and the concentration of conjugated peptides in column fractions was measured at 280 nm absorbance using a spectrophotometer.

Phage ELISAs

To analyse the binding of peptide phage clones, ELISAs were performed by coating a microtiter plate overnight at 4°C (Nunc, Maxisorp) with 100 µl/well of 10 µg/ml of purified rabbit or human polyclonal antibodies in PBS. Coated wells were washed twice with PBS and blocked with 10% blotto (milk powder/PBS) for 2 h. Phage dilutions (100 µl) were prepared in PBS, transferred in duplicate to the coated blocked wells and incubated for 1 h on a plate shaker. Wells were washed five times with PBS containing 0.1% Tween 20 (PBST) and 100 µl of anti-M13 antibody conjugated to horseradish peroxidase (HRP, Pharmacia) at 1/5000 dilution was added to each well. After 1 h incubation and washing as above, bound phages were detected with *o*-phenylenediamine substrate (Sigma).

Antigen ELISAs

ELISAs were performed using plates coated with crude EBV, p18, EBNA, EA-D and VCA antigens (pre-coated and blocked plates were kindly provided by PanBio Ltd, Brisbane). Serum dilutions were prepared in fish gelatin diluent [2% fish gelatin (Sigma), 1% BSA (CSL), 1% Tween 20 in PBS] and incubated for 1 h on the plate shaker. The plate was washed as above and sheep anti-human IgM-HRP (Chemicon) at 1/5000 dilution was added for 1 h. After a final wash step, binding was detected with 3,3′,5,5′-tetramethylbenzidine substrate (Sigma).

BSA-conjugated peptide ELISAs

For ELISAs using peptides conjugated to BSA, a procedure similar to our previous study was used (Casey *et al.*, 2006). One hundred microlitres per well of peptide conjugates were coupled to maxisorp plates at 5 µg/ml overnight at 4°C. Plates were washed three times with PBS and serum sample dilutions in PBST were added to the blocked wells in duplicate. The remainder of the procedure was performed as above, using goat anti-rabbit-HRP (Chemicon) at 1/2000 dilution.

All ELISAs were performed in duplicate or triplicate and assays were repeated to establish reproducibility of results.

Results

Reactivity of rabbit anti-EBV antibodies

The rabbit immunised with an extract of EBV was shown to have a high titer of antibodies (>1/1000) to p18, crude EBV and VCA antigens as shown in Fig. 1A, when compared with the low reactivity of the pre-bleed fraction to these antigens. The IgG fraction of the antisera was affinity-purified using protein G resin and this preparation was used for immunopanning.

Characterisation of EBV human sera

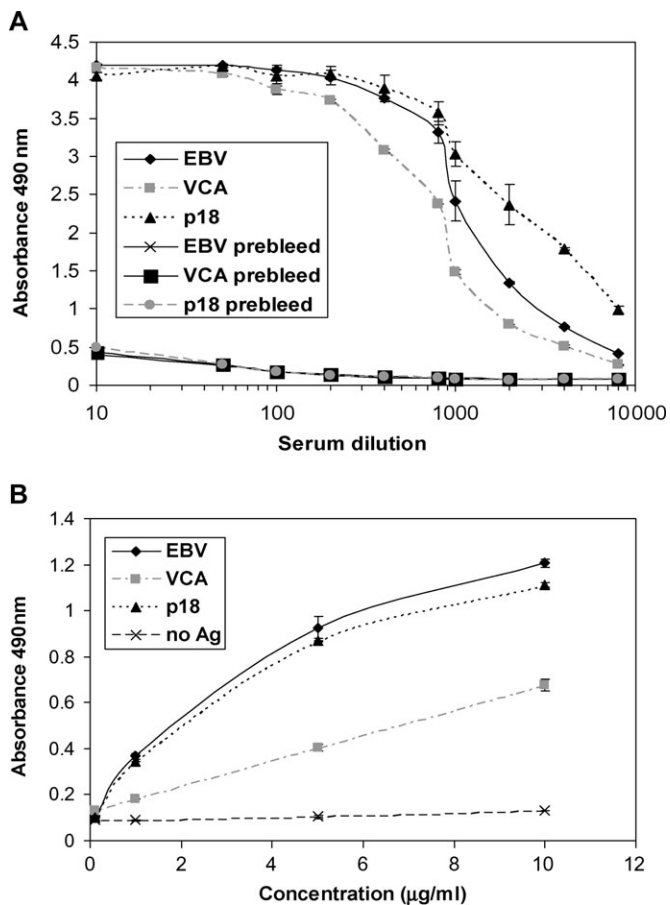Human serum from an individual with a high titer of EBV IgG antibodies (OD units in ELISA >2.0) confirmed using

Fig. 1. Characterisation of EBV immune sera. (**A**) ELISA showing the antibody titer of rabbit serum to crude EBV, VCA and p18 antigens before and after immunisation. (**B**) Specificity of human serum tested positive on a commercial EBV test that has been affinity purified using Protein G. ELISA showing reactivity of this human IgG to EBV antigens, crude EBV, VCA, p18 compared with wells coated with no antigen (no Ag).
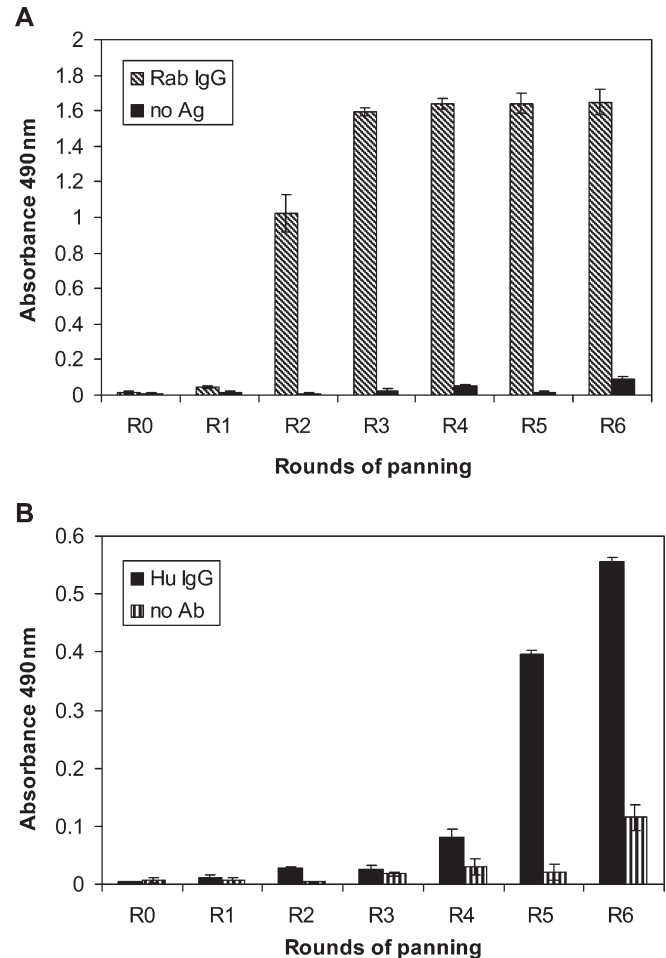

Fig. 2. Selection of phage clones that are recognised by (**A**) rabbit anti-EBV IgG (Rab IgG) and (**B**) human anti-EBV IgG (Hu IgG). The reactivity of selected phages from each round of panning (R) is shown by ELISA. Error bars indicate the ranges of individual values.

an EBV diagnostic kit (PanBio Ltd) was purified using protein G resin. The reactivity of the purified antibodies is shown in Fig. 1B. There was high binding to crude EBV and

Table I. Peptide sequences of clones selected after 4, 5 or 6 rounds of panning on immune rabbit and human EBV IgG

Amino acid sequence	
D G P S Y H V A F K N S R G L R H S	H1 [a]
N G A L Y P R F F P D Y S I L M F P I I	Eb1 [a]
D Q F A Q A Y R G D R N F F N E L T S T	Eb2 [a]
R Q F S K F K D A S D R Y G N Y L H F F	Eb3 [a]
S S S I K I W N K L G W N T V I A G T R	Eb4 [a]
F V N A F Q N A N F M R P R E L F A L A	Eb5
S A N L N F F S P D F G L Y T P N A S A	Eb6
A I T C A H T L S I K S R R C Q Y V F K	Eb7
A A S Y A S R T V G F A S V Y W F S R P	Eb8
R L R G D Y N V G P I R F G W P V A P N	Eb9
M S D F D R K V Y T F N F I T D P Q H L [b]	Eb10
G V T D F D F K V F S S T F P K I F L S [b]	Eb11
T P N T V R D F Y Y N V S L P S Y M L I [b]	Eb12
G G W Y S F D S P Y L M S I T E M R L R	Gp125 [c]
Y T D S S M A V T L M K F A S N F L F	F1 [c]

Bold and underlined areas represent areas of homology.
[a] Indicates peptides selected for further study.
[b] Indicates binding to pre-immune sera.
[c] Indicates peptides Gp125 and F1 described previously (Casey *et al.*, 2006).
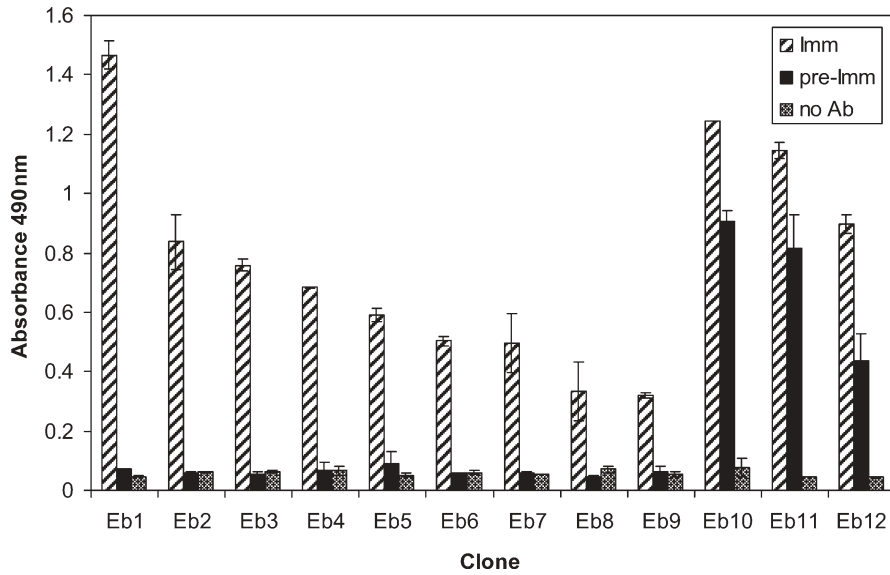
Fig. 3. Reactivity of rabbit EBV IgG individual phage clones. Clones isolated from rounds 4–6 selected on rabbit EBV IgG were analysed for reactivity with immune and pre-immune IgG by ELISA. Clones Eb1–4 with the highest binding to immune serum were selected for further study.

p18 antigens slightly lower binding to VCA antigen, when compared with ELISA wells containing no antigen. This antibody preparation was used for immunopanning.

Selection of peptide mimotopes

Peptides mimicking epitopes of anti-EBV rabbit IgG and anti-EBV human IgG were isolated by screening a 20 amino acid random linear peptide library (AdLib 1) using multiple rounds of panning. For selection of phage binding to rabbit EBV IgG, an increased number of bound phage was detected after the second round of panning with a further increase in round 3 and a plateau in binding in rounds 4, 5 and 6 (Fig. 2A). For immunopanning on human EBV IgG, enrichment in binding was observed after the fourth round of panning, indicated by an increase in binding to antigen and this increased further in subsequent rounds 5 and 6 (Fig. 2B).

Sequences of phage clones

DNA sequences of 10 clones from each round of panning with a high ELISA signal, i.e. rounds 4, 5 and 6, are summarised in Table I. Twelve different sequences with high reactivity with the rabbit EBV IgG fraction were identified, whereas only one sequence from a total of 30 clones was isolated that showed the specificity for the human EBV IgG preparation. No consensus sequence was observed; however, a small amount of homology for some of the sequences is shown in Table I. For example, Eb10 and Eb11 shared a similar region 'D **F D** (**R/**F) K V' and Eb12 contained 'F D R' (in reverse orientation). This sequence of amino acids is contained within the same area of homology shown in bold type in the reverse orientation. In addition, sequences Eb4 and Eb7 also had a small area of homology, 'S I K'. Furthermore, 4/12 sequences contained an 'F F' motif.

Characterisation of individual phage clones

Individual phage clones were analysed for reactivity with pre-immune and immune EBV rabbit IgG antibodies (Fig. 3). Only 3/12 phage clones were reactive with the pre-immune IgG indicating the remaining nine clones bind to

EBV-specific antibodies in the immune sera. The two clones Eb10 and Eb11 described above with a similar area of homology 'D F D (R/F) K V' were both recognised by antibodies
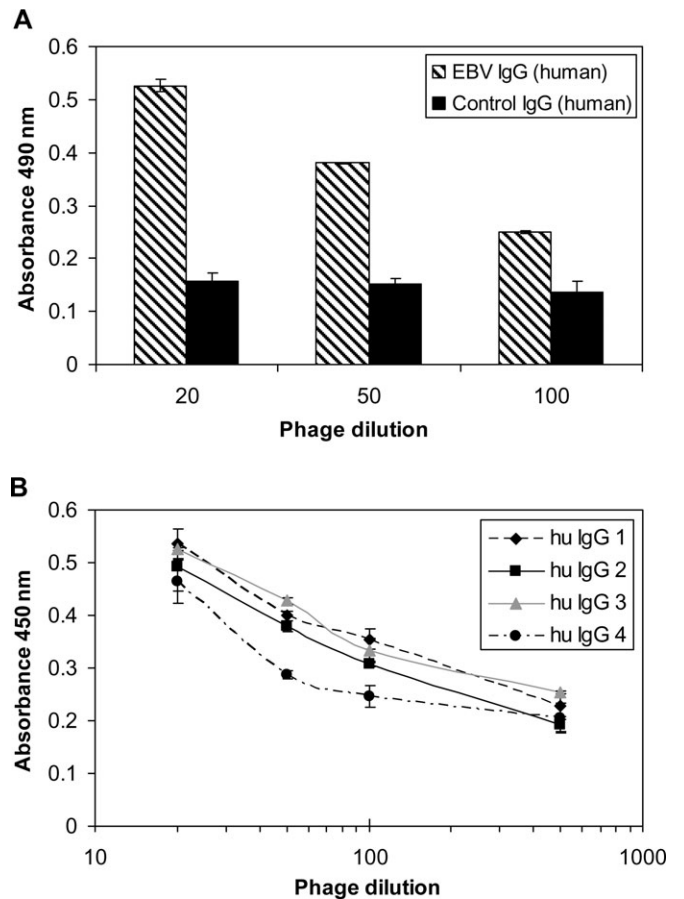

Fig. 4. Reactivity of isolated phage clone (H1) selected by panning on human EBV IgG with the same IgG and a non-specific human IgG preparation (**A**). H1 is recognised by four different affinity purified EBV-seropositive IgGs (**B**), indicating specificity for a common epitope typically present in individuals infected with EBV. Bars show the mean ELISA signal of duplicate wells and the bars indicate +/− errors.
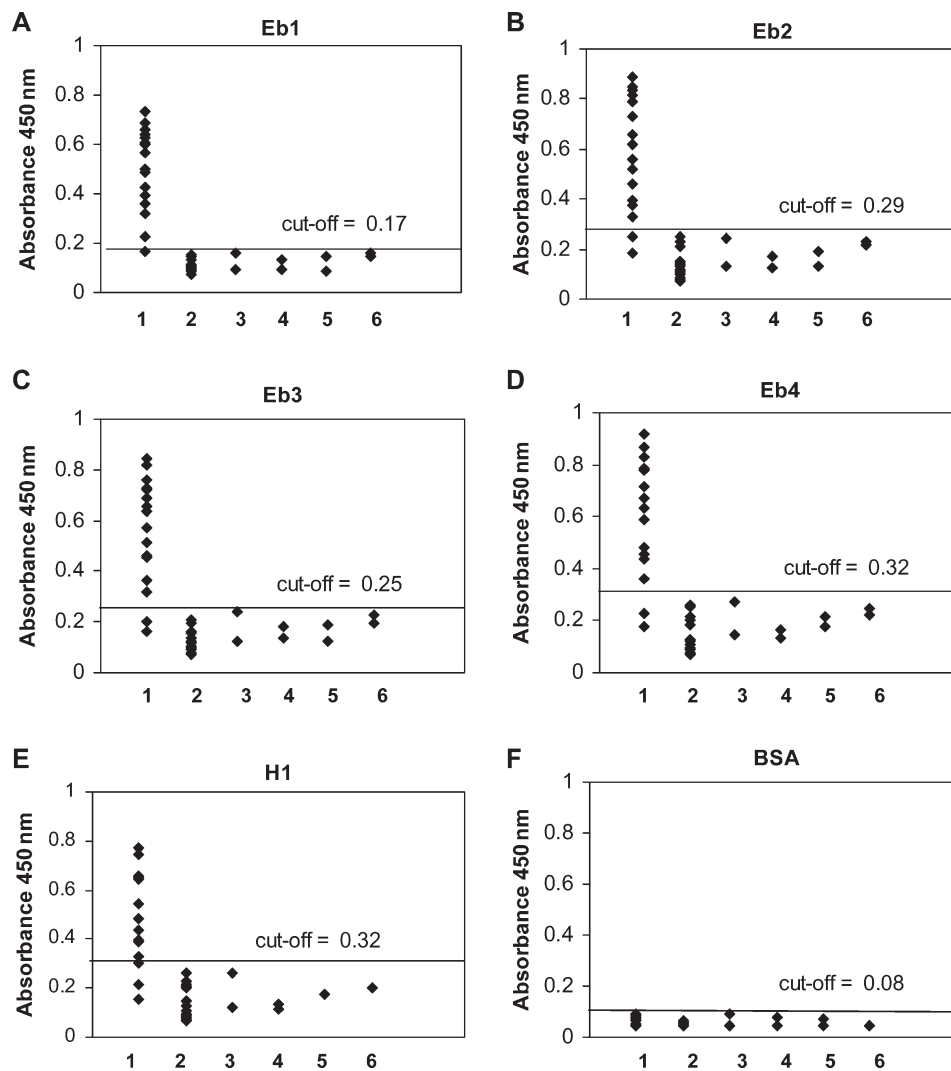
Fig. 5. Evaluation of peptides Eb1–4 and H1 coupled to BSA as EBV diagnostic reagents. Human serum ($n = 40$) previously analysed using a diagnostic test for VCA IgM was allowed to react with the peptides and the bound IgM antibodies were detected using anti-human IgM HRP. The absorbance readings for 1 (positive), 2 (negative) and putative cross-reactive sera for 3 (Parvo), 4 (HSV), 5 (CMV) and 6 (RF) are plotted for (**A**) Eb1, (**B**) Eb2, (**C**) Eb3, (**D**) Eb4, (**E**) H1 and (**F**) BSA, respectively. The cut-off value is defined as the mean of the negative population +3SD indicated by a solid horizontal line; since there were no false positives, the specificity for each mimotope was 100%.

in the pre-bleed sample, Eb12 containing the sequence 'F D R' (in reverse orientation) was also recognised by the pre-immune sera, therefore, indicating these clones are representing non-EBV-specific epitopes. The four clones with the highest reactivity with rabbit EBV immune sera (Eb1–Eb4) were selected for further study.

The single clone selected from the random peptide library (H1) with high reactivity with the human IgG EBV preparation was shown to have low reactivity with a control IgG (Fig. 4A). This observation suggests EBV-specific antibodies are reactive with H1 phage. Importantly, antibodies in the serum of purified EBV-positive individual sera from four individuals were reactive with H1, indicating the specificity with an epitope common to antibodies present in each of these positive sera (Fig. 4B).

Eb1–4 and H1 phage clones were not recognised by mAbs (gp125, F1, A2 and A3; data not shown) described in our previous study (Casey *et al.*, 2006), suggesting that we have selected novel peptides that do not mimic similar epitopes of these antibodies and therefore should represent

different epitopes that are perhaps specific to those induced during a natural EBV infection.

Peptide-BSA conjugates as diagnostic antigens

To analyse the potential of the peptides to behave as antigen mimics, their ability to react with IgM antibodies from individuals infected with EBV was assessed. In our previous study, we demonstrated that the sensitivity of detection was greatly improved when the peptides were coupled to a carrier molecule such as BSA prior to immobilisation onto a solid surface (Casey *et al.*, 2006). This strategy was adopted to test peptides Eb1–4 and H1. A set of 40 clinical samples that were classified as EBV seropositive ($n = 16$), seronegative ($n = 16$) or potentially cross-reactive sera ($n = 8$) were assessed for reactivity with Eb1–4 and H1 peptides individually. The cut-off level was defined as the mean optical density of the seronegative samples plus 3 standard deviations shown as a line on the graphs in Fig. 5. Readings above this level were defined as positive and below this level negative. The same set of samples were analysed on BSA

alone and these values were subtracted from the peptide-BSA conjugate readings and the corrected absorbance readings were plotted individually for our new peptides Eb1–4 and H1 in Fig. 5. There was a clear difference in the detection of seropositive antibodies by all the peptides (Fig. 5A–E) compared with the analysis of BSA alone (Fig. 5F), with the majority of absorbance readings above the cut-off level. We compared the ability of our panel of peptide mimotopes to be recognised by antibodies in the same set of seropositive samples in Fig. 6A and the sensitivity of detection is shown in Fig. 6B. We also included F1 and Gp125 mimotopes specific for two mAbs in our previous study (Casey *et al.*, 2006). Of the peptides identified from polyclonal sera Eb1, Gp125 and F1 had the highest sensitivity (94%). Slightly lower sensitivity was observed for Eb2, 3 and 4 (88%) and H1 peptide had the lowest sensitivity (81%) as summarised in Fig. 6B. The sensitivity of F1 and Gp125 was similar to that produced by the mimotopes selected in our previous study, 95% for F1 and 92% for Gp125.

We also considered which seropositive EBV samples contained antibodies that did not recognise the panel of peptides, i.e. false-negative readings, listed in Fig. 6B. The antibodies in serum 1 (s1) were unreactive with all of the peptides identified in this study, s2 was not reactive with Eb3, Eb4 and H1 and s3 was unreactive with H1. Gp125 and F1 that were selected in our previous study were recognised by s1, 2 and 3; however, two different serum samples (s4 and 5) did not recognise F1 or Gp125, respectively. This demonstrates that individual peptides are not recognised by all EBV antibodies and confirms that different peptides are required to represent different epitopes. Therefore, a combination of Eb1 peptide F1 and Gp125 peptides could be recognised by antibodies present in all this set of EBV clinical samples resulting in 100% sensitivity.

For the samples defined as EBV-seronegative, there were no readings above the cut-off level and therefore no false positives, resulting in 100% specificity. In addition, there were no absorbance readings above the cut-off levels for the potentially cross-reactive serum samples, inferring that the peptides identified in this study have high specificity for EBV antibodies.

Discussion

We have developed a library screening approach to select peptides that can substitute for the cognate antigen in assays used in the diagnosis of acute EBV infection. EBV mimotopes were isolated from a phage-displayed peptide library by screening purified antibodies derived from polyclonal human sera and hyperimmune rabbit sera. This novel approach enables the simultaneous selection of peptides that mimic different epitopes with no prior knowledge of the native antigen and is therefore more rapid than selections using mAbs.

The strategy to select disease-specific epitope mimics using immune sera has so far been largely unexplored as many previous studies in this field have employed mAbs to neutralising or immunodominant epitopes. However, a few reports have described the use of patients' sera to identify peptide mimics. Polyclonal sera has been used to select peptides for Lyme disease (Mathiensen *et al.*, 1998), Hepatitis C
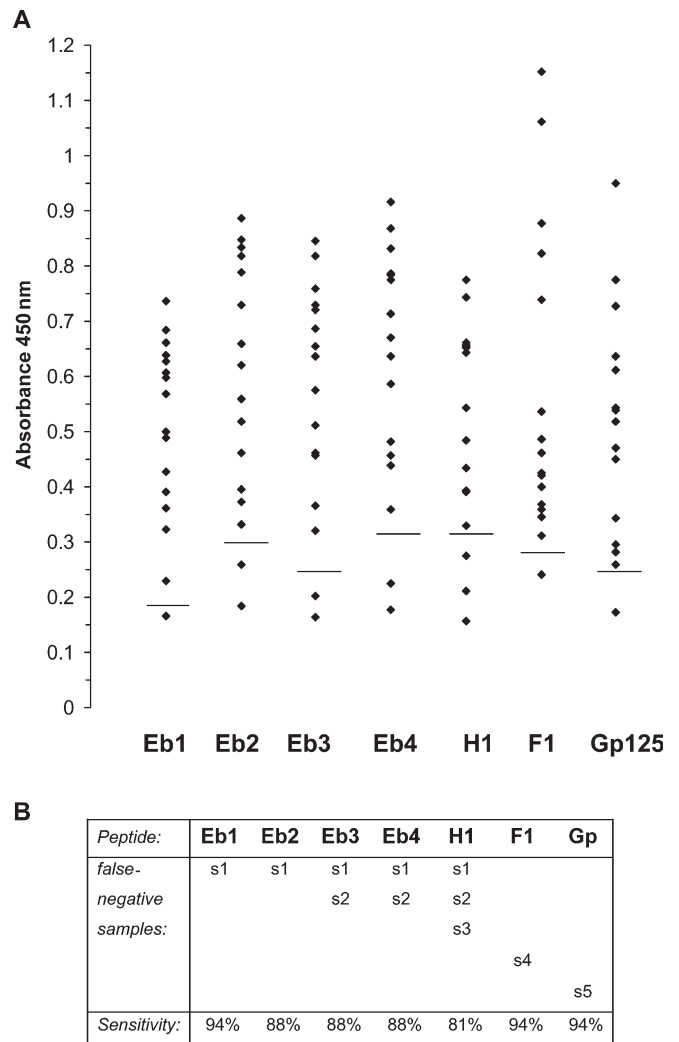
Fig. 6. Comparison of the reactivities of our panel of mimotopes Eb1–4, H1, F1 and Gp125 conjugated to BSA with EBV IgM-positive sera ($n = 16$) absorbance values are plotted and the cut-off levels are depicted by a horizontal line in (**A**). (**B**) Summary of the false-negative results from the 5/16 serum samples seropositive for IgM EBV and the overall sensitivity for each mimotope for diagnosis of EBV IgM antibodies.

Peptide:	Eb1	Eb2	Eb3	Eb4	H1	F1	Gp
false-negative samples:	s1	s1	s1	s1	s1		
			s2	s2	s2		
					s3		
						s4	
							s5
Sensitivity:	94%	88%	88%	88%	81%	94%	94%

virus core protein (Prezzi *et al.*, 1996) and Hepatitis A virus (Larralde *et al.*, 2007) and led to the identification of disease-related peptides by screening large numbers of clinical sera from immune and non-immune individuals with no prior knowledge of the target antigen (Folgori *et al.*, 1994). More recently, phage display peptide libraries were used to validate a specific serological marker and identify the native antigen by screening antibodies purified from whole serum derived from prostate cancer patients (Mintz *et al.*, 2003).

In this study, we have identified several peptides that can be used individually for detection of natural antibodies produced by patients recently infected with EBV. All the peptides demonstrated high specificity (all 100%) and sensitivity (94%, 88%, 88%, 88% for Eb1–4 and 81% for H1). As we have noted previously, these peptides have no obvious homology to EBV antigens and further studies should be carried out to identify their corresponding antigens. In addition, the peptides had low reactivity with negative and putative cross-reactive sera, indicating the high specificity of small peptides for serological diagnosis compared with a complex antigen

which contains many unwanted epitopes. When the peptides were used in combination, a greater sensitivity was observed (up to 100%), indicating this is a requirement for complete coverage of pathogen-specific antibodies in the sera of patients.

The aim of this study was to select peptides that mimic the most abundant and/or immunodominant epitope present during a recent infection with EBV. We chose to purify an individual serum rather than a pool of high titre patient samples. If a pool was used, these epitopes may have been diluted making it more difficult to isolate a peptide reactive with the antibody/s generated by this immunodominant epitope. We have shown here (Fig. 4) that the peptide mimotope H1 was recognised by antibodies in four different clinical samples, proving that antibodies derived from an individual seropositive serum can produce a pathogen-specific peptide mimic. In order to extend the coverage of diagnostically relevant epitopes, further selections could be carried out using a pool of high titer seropositive EBV samples to decipher whether more peptides could be selected simultaneously and similarly to the data we have shown in this study using polyclonal sera from rabbits immunised with EBV.

In conclusion, we describe in this study a panel of EBV peptide mimotopes when used in combination are recognised by the whole repertoire of antibodies typically produced after acute infection with EBV. This methodology could be applied to many diseases and may provide novel reagents for diagnosis and prognosis of diseases and reveal information regarding unknown pathogenic agents.

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