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A general strategy to identify mimotopes of pathological antigens using only random peptide libraries and human sera

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A strategy to identify disease-specific epitopes from phage-displayed random peptide libraries using human sera is described. Peptides on phage (phagotopes) that react with antibodies present in patient sera are purified from $>10^7$ different sequences by affinity selection and immunological screening of plaques. Disease-specific phagotopes can be identified out of this pool through an 'antigen independent' procedure which avails itself only of patient and normal human sera. Using this strategy, we have selected antigenic mimics (mimotopes) of two different epitopes from the human hepatitis B virus envelope protein (HBsAg). We could show that a humoral response to these mimotopes is widespread in the immunized population, suggesting that the strategy identifies phagotopes that have a potential role as diagnostic reagents. Immunization of mice with the selected phagotopes elicited a strong specific response against the HBsAg. These results open new inroads into disease-related epitope discovery and provide the potential for vaccine development without a requirement for the use of, or even information about, the aetiological agent or its antigens.

Key words: antigenic mimic/hepatitis B surface antigen/ human sera/phage peptide library

Introduction

The recombinant DNA approach toward the generation of diagnostic reagents and vaccines requires detailed information about the antigens, as well as about their genes. This constitutes a major limitation in the study of infectious, allergic or autoimmune diseases whose aetiological agent is unknown. However, tracks of the aetiological agent of these diseases are imprinted in patient sera as a specific immune response. A method for decoding such information without knowledge of the aetiological agent or its antigen(s) could greatly enhance the possibilities for the diagnosis and prognosis of these diseases.

Random peptide libraries displayed on phage (RPL) can be operationally defined as 'all purpose' libraries as they have been used as a source of ligands to ligates interacting with linear peptides or folded protein domains, and even with non-proteinaceous molecules (Cwirla *et al.*, 1990; Devlin *et al.*, 1990; Scott and Smith, 1990; Felici *et al.*, 1991, 1993; Oldenburg *et al.*, 1992; O'Neil *et al.*, 1992; Scott *et al.*, 1992; Stephen and Lane, 1992; Blond-Elguindi *et al.*, 1993; Hammer *et al.*, 1993; Luzzago *et al.*, 1993). The ligands do not necessarily resemble the natural ones, but they mimic their binding properties (mimotopes). For this reason, RPL can be used as a source of epitopes not derived from the natural antigen, but selected because they fit the binding antibodies.

A more general application of the phage display technology would be the use of polyclonal antibodies in the screening of RPL. The identification of disease-specific mimotopes using sera from patients could provide a set of novel reagents useful for the diagnosis of the disease. Furthermore, in those cases where the humoral immune response is protective, disease-related mimotopes displayed on phage (phagotopes) could represent a step in the development of acellular vaccines.

In many diseases in which the aetiologic agent is unknown, disease-specific phagotopes could be used to define common features in the immune response of different individuals to the same aetiological agent: its 'immunofootprint'. In these cases, the selected phagotopes could prove to be an invaluable tool for the identification of the aetiological agent.

In this report, we describe a strategy to identify disease-specific phagotopes from RPL which does not require detailed information about the natural antigen(s), but avails itself of clinically characterized sera from patients and normal individuals.

Results

Screening of a random peptide phage library using human sera

With the aim of developing a general strategy for screening phage libraries with polyclonal antibodies, we have used as a model system sera from both human hepatitis B virus envelope protein (HBsAg)-immunized individuals (defined throughout this paper as disease-specific or positive sera) and non-immunized individuals (defined as normal or negative sera). This choice was based mainly on the knowledge that either recombinant HBsAg or a synthetic peptide reproducing part of its amino acid sequence is able to elicit a protective immune response (Purcell and Gerin, 1985). HBsAg immunization thus offers an opportunity to assess the feasibility of using selected phagotopes as immunogens and potential candidates for vaccine development.

Most of the technical difficulties encountered in screening molecular repertoires using polyclonal antibodies are related to the nature of the ligate. In fact, in the case of sera, the selector is a large collection of different molecules that display a wide range of unknown affinities and concentrations. Owing to their polyspecificity, human sera

Table I. Affinity selection and immunological screening

	Affinity selection	Immunological screening			
		CB	mAbs	PM	RT
Unselected library		1.4×10^{-3}	5.1×10^{-5}	ND	ND
Mock-selected phage	1.7×10^{-7}	1.1×10^{-3}	ND	ND	ND
CB+	1.0×10^{-6}	1.3×10^{-2}	2.0×10^{-4}	3.3×10^{-3}	2.9×10^{-3}

Yields from affinity purification in the absence (mock-selected phage) or in the presence of serum (CB^+) are reported in the first column as ratios between the number of phage eluted after the affinity selection and the number of input phage. In the next four columns are reported the results of the immunological screening of plaques from the unselected library, the mock-enriched phage and the CB-enriched phage (CB^+) using three disease-specific sera (CB, PM and RT) and a mixture of the two anti-HBsAg mAbs RFHBS6 and BA1 (see Materials and methods). Numbers refer to the ratio of positive over total plated phage

will, in addition, enrich phagotopes which are not disease specific. For these reasons, the approaches previously utilized for the selection of ligands using homogeneous ligates such as monoclonal antibodies (mAbs; Smith, 1991; Christian *et al.*, 1992; Scott *et al.*, 1992) are not suitable for the screening of RPL using polyclonal antibodies. We repeatedly attempted to subtract phagotopes reacting with non-disease-specific antibodies by affinity selection using negative sera, but in no case were we able to significantly deplete the library. This is presumably due to the low efficiency of phagotope selection (P.Monaci and A.Nicosia, unpublished data). We therefore devised a different experimental strategy that can be divided into three steps: (i) affinity selection of a random peptide library with a positive serum; (ii) screening of the enriched phage population for epitopes also recognized by a second positive serum; (iii) further screening of the selected phagotopes using large panels of positive and negative sera.

A library of nonapeptides fused to the major coat protein pVIII (pVIII9aa-cys; Luzzago *et al.*, 1993) was chosen as a source of epitopes because in this system up to several hundred peptides are displayed on each phage particle. Moreover, most of the peptides are displayed as disulfide bridge-constrained loops. These features represent a significant advantage over other display systems in terms of effective concentration of ligands (Felici *et al.*, 1991; Luzzago *et al.*, 1993; McLafferty *et al.*, 1993).

Polystyrene beads coated with a sample from a patient serum (CB) were used to affinity select 2×10^{11} library phage particles. This corresponds to >1000-fold the actual complexity of the library. About 4×10^5 phage were recovered after washing non-reacting phage and elution of bound particles (Table I). Similar results were obtained by performing the affinity selection with three other positive sera (data not shown). Mock selection in the absence of serum yielded 10 times fewer phage (Table I), indicating that phage clones reacting with serum antibodies were specifically enriched by the above selection procedure.

In the case of monospecific ligates, several rounds of affinity purification were used to eliminate background phage (Smith, 1991; Scott *et al.*, 1992). However, in the absence of any information on the concentration and affinity of the disease-specific antibodies, it is not possible to predict whether multiple selection steps would effectively favour enrichment of disease-specific versus other phagotopes. Furthermore, during amplification, any difference in the growth rate among the various phage clones might introduce a biological selection unrelated to that obtained by affinity. To overcome these limitations and to allow rapid identification of positive clones among large numbers (10^5-10^6) of phage, we developed a method for the immunodetection of phagotopes on filters using crude sera.

Specific detection of phage fusion proteins with sera in Western blotting and colony blotting has so far been hampered by the high cross-reactivity of human sera with bacterial and/or filamentous phage proteins (Tsunetsugu-Yokota *et al.*, 1991; Minenkova *et al.*, 1993; P.Monaci and A.Nicosia, unpublished data). To lower the background and to increase the signal to noise ratio, we explored the possibility of immunoscreening phage clones plated as plaque-forming units (p.f.u.). Since the pVIII library that we used was constructed in phagemid vectors, p.f.u. were obtained by plating, on a competent bacterial lawn, phagemid-containing bacteria infected by a helper phage. To set up the conditions for the immunodetection, we chose two phage clones previously selected from the library using a human serum. These two phagotopes were found to react with human antibodies present in many sera from patients and normal individuals by enzyme-linked immunosorbent assay (ELISA) using caesium chloride-purified phage as coated antigen (A.Folgori, unpublished data). The two phage clones were plated as p.f.u., transferred onto nitrocellulose filters and probed against different human sera. Specific reactions were detected which mirrored the data obtained with these clones in ELISA using the same sera. No signals were detected by incubating the sera with a filter containing wild-type phage plaques (data not shown).

Using this procedure, we analysed the serum-enriched phage pool (CB^+) for the frequency of clones that specifically reacted with serum antibodies. Phage from the unselected library and a mock-enriched library were also screened as a control. The results of the experiment showed that the number of clones reacting with the probe after one cycle of selection with the CB serum was 10-fold that of the unselected library or the mock-enriched phage pool (Table I). These data confirmed that phage clones interacting with serum antibodies were selected from the library during the affinity step.

To verify whether affinity selection with the HBsAg immune serum also enriched phage bearing epitopes reacting with anti-HBsAg antibodies, samples from the unselected library and from the enriched library were immunoscreened with a mixture of two anti-HBsAg mAbs of different specificity (see Materials and methods). As shown in Table I, selection with the CB serum resulted in an increase of phage which are recognized by anti-HBsAg mAbs.

Identification of phage-displayed disease-specific epitopes

A major goal of the entire strategy was to increase the efficiency of selection of disease-specific clones and to identify them without using the antigen. For this purpose, we next used more than one patient serum in the immunoscreening step. In fact, screening the affinity-selected phage with a patient serum different from that used for the enrichment allows only those phage displaying epitopes recognized by antibodies of 'common' specificity between the two sera to be identified. Among the common phagotopes there should be a high percentage of disease-specific ones. A phagotope could then be considered specific for disease antibodies on a statistical basis by further screening using many different positive and negative sera.

The affinity-selected phage (CB^+) were plated as p.f.u. and immunoscreened with two different positive sera: RT and PM. The number of plaques detected by each of the two sera was lower than that observed by screening with the CB serum (Table I). Similar results were obtained by performing affinity selection of the library and immunoscreening of the enriched phage with other pairs of sera (data not shown). These data indicated that in our experimental system a core of antibodies of 'common' specificity ($\sim 25\%$) between the antibody repertoires of two genetically unrelated individuals is always identified. Twenty-five positive plaques from each of the two screenings were randomly chosen, purified and re-screened. Fifteen phage were confirmed to be recognized by antibodies present in both the enriching and screening sera.

We then performed a counter-screening of the double-positive phage using many negative sera to eliminate phagotopes that are not recognized by disease-specific antibodies. In order to do this in a more sensitive and quantitative way than by plaque immunoscreening, we developed a rapid and sensitive ELISA assay for the detection of pVIII-displayed epitopes with human sera using crude phage supernatants from infected bacterial cultures. Heretofore, recognition of epitopes with phage-coated plates in ELISA using human sera has been obtained only by caesium chloride purification of phage particles (A.Folgori, unpublished data). This causes the screening of large numbers of clones by ELISA to be very laborious, if not impossible. To solve this problem, we used an anti-M13 mAb recognizing the pIII coat protein (YOPH57D.1; L.Dente, G.Cesareni, G.Micheli, F.Felici, A.Folgori, A.Luzzago, P.Monaci, A.Nicosia and P.DelMastro, submitted) to tether phage on the surface of multiwell plates from crude supernatants of cultures of infected bacteria (see Materials and methods). In this way the double-positive phage were tested for their reactivity to 18 different sera from normal individuals. Most phage were recognized by the normal sera and were hence classified as non-specific for the disease (Figure 1A). It is worthy of note that 10 of these phage reacted with at least seven different negative sera, while the remaining three were recognized by more than two negative sera. Only two phage (ϕ13 and ϕ41) did not react with any of the negative sera (Figure 1A).

As the latter phage were selected by virtue of their reactivity with only two disease-specific sera (CB + RT and CB + PM, respectively), we determined the frequency of their recognition by a larger number of positive sera (20) in ELISA. As shown in Figure 1B, ϕ13 reacted with 14 positive sera, while ϕ41 was recognized by antibodies present in 13 of the tested samples. The frequency with which each phagotope was recognized by the negative sera (0/18) was remarkably different and statistically very significant (the probability that the observed frequency distributions of each phagotope between positive and negative sera are statistically homogeneous is <0.005). On this basis, ϕ13 and ϕ41 were considered disease-specific phagotopes.

Serum-selected phagotopes represent two different epitopes

Sequencing the inserts of the selected clones revealed some homology of ϕ13 with amino acids 121–127 of the HBsAg located in one of its most antigenic sites (*d/y* determinant region; Valenzuela *et al.*, 1980; Figure 1C). In contrast, the peptide displayed by ϕ41 did not show any similarity with the natural antigen. In an independent experiment, two additional disease-specific phagotopes have been identified (ϕ17 and ϕ30) whose amino acid sequences are homologous to that of ϕ41 (Figure 1C). ϕ17 and ϕ30 also displayed a pattern of reactivity with patient sera similar to that of ϕ41 (data not shown). These data suggested that ϕ17, ϕ30 and ϕ41 mimic the same epitope, and ϕ13 a different one. To prove this hypothesis, we performed cross-inhibition assays between the different clones with the CB serum in ELISA. Each of the three homologous clones abolished recognition of the other two by serum antibodies when used as competitors (Figure 2). No inhibition was observed either when ϕ13 was used to block recognition of ϕ17, ϕ30 and ϕ41 by CB antibodies, or in similar reactions with ϕ13 as target and the other three phage as competitors (Figure 2). These results allowed us to conclude that the serum-identified phagotopes can be grouped into two classes recognized by antibodies of different specificity.

Disease-specific epitopes selected with human sera are HBsAg immunogenic mimics

If the peptides displayed by the disease-specific phagotopes reproduce immunogenic HBsAg epitopes, they might also be immunogenic mimics of the natural antigen. To test this hypothesis, we have studied the immunogenicity of one representative from each of the two classes of mimotopes in mice. We used purified phagotopes as immunizing agents because filamentous phage are good immunogens in different animal systems (de la Cruz *et al.*, 1988; Greenwood *et al.*, 1991; Minenkova *et al.*, 1993; Willis *et al.*, 1993). ϕ13 was injected into six animals (three BALB/C and three C57Bl), while four mice (one BALB/C and three C57Bl) were immunized with ϕ41. All animals developed a strong and comparable response against wild-type phage (data not shown). All sera from mice immunized with phagotopes 13 and 41 showed an anti-HBsAg response ranging from 3- to 12-fold the background signal observed using the serum from a mouse immunized with wild-type phage (Figure 3A and B). The response induced by ϕ13 was, on average, greater than that obtained by immunization with ϕ41. Differences in the extent of the reaction with the antigen were also observed between sera from individual animals injected with the same phagotope, but this variability did not correlate with the genetic strain (Figure 3A and B). To prove that the response to HBsAg was specific, we performed competition assays with the purified phagotopes. HBsAg and wild-type

A

	Φ3	Φ4	Φ13	Φ19	Φ20	Φ24	Φ26	Φ29	Φ32	Φ34	Φ35	Φ37	Φ41	Φ43	Φ44
AM	**2.00**	**0.32**	-0.01	-0.02	0.14	**0.82**	**0.98**	**0.41**	**0.49**	**0.44**	**0.43**	0.01	-0.01	**0.33**	**0.37**
EP	**2.26**	0.04	0.00	0.02	0.03	**0.49**	**0.55**	0.02	**0.22**	**0.55**	0.01	0.09	0.03	**0.43**	0.02
FT	**0.15**	**1.19**	-0.01	**0.30**	**0.61**	-0.02	**1.58**	**0.18**	**0.18**	**0.17**	0.14	0.02	0.00	0.06	0.09
AT	**2.21**	0.06	0.01	0.03	0.04	0.03	**0.16**	0.13	**0.43**	**0.56**	0.12	**0.23**	0.01	**0.79**	0.12
TP	**1.90**	0.02	0.00	0.04	0.01	**0.47**	-0.01	-0.02	-0.02	0.01	-0.01	0.00	0.00	0.01	-0.01
CFI	**1.88**	**0.74**	-0.01	0.10	0.11	**0.84**	**1.72**	**0.89**	**0.18**	**0.92**	**0.77**	**0.34**	0.03	**0.99**	0.05
RBA	0.02	**0.22**	0.00	0.13	**0.15**	0.09	0.07	0.05	0.14	**0.59**	0.07	**0.31**	0.03	0.10	0.01
CN	**0.19**	**0.23**	0.00	0.01	0.03	**0.74**	**1.58**	**0.45**	0.06	0.07	**1.91**	0.03	0.03	0.04	0.04
AW	0.04	**0.49**	0.00	**0.18**	**0.17**	**0.23**	0.04	0.00	0.04	**1.35**	0.00	**1.48**	0.01	**0.22**	0.12
VA	0.06	**0.16**	0.00	0.02	0.03	0.05	**0.79**	**0.50**	-0.02	0.01	**0.73**	0.01	0.00	0.00	0.00
KW	0.05	0.04	0.00	0.01	0.01	0.02	**0.56**	0.00	**0.27**	**1.03**	0.00	**0.50**	0.00	**0.45**	-0.02
RR	**0.18**	**0.17**	0.01	**0.20**	0.13	0.02	**0.44**	**0.27**	0.07	**0.87**	**0.38**	0.13	0.00	**0.25**	0.08
CFA	**0.70**	**0.50**	0.00	0.14	0.03	**0.51**	**0.42**	**0.19**	0.00	**1.18**	**0.57**	**0.46**	0.01	**0.37**	-0.02
AN	**1.54**	**1.18**	0.07	0.14	**0.21**	**0.79**	**0.48**	**0.21**	0.10	**0.97**	**0.22**	**0.49**	0.04	**0.19**	0.01
FF	0.03	0.00	0.00	0.00	0.01	0.00	**0.48**	-0.02	0.00	**0.17**	0.06	0.03	0.03	0.02	**0.26**
RB	-0.05	-0.09	-0.01	-0.07	-0.03	-0.06	0.03	-0.01	0.11	**1.19**	-0.08	0.00	-0.02	-0.03	**0.90**
MN	**2.22**	**0.15**	0.01	-0.03	-0.03	**1.67**	**0.22**	**0.16**	**0.27**	0.00	0.06	-0.02	0.00	-0.01	-0.02
PC	-0.11	-0.02	0.06	-0.10	-0.12	-0.05	0.03	**0.63**	-0.02	0.09	**0.78**	0.01	0.01	0.03	0.07
recognition frequency	11/18	11/18	0/18	3/18	4/18	9/18	13/18	10/18	7/18	13/18	8/18	7/18	0/18	9/18	3/18

B

	Φ13	Φ41
CB	**1.89**	**0.62**
RT	**0.49**	**0.87**
PM	**0.51**	**0.49**
ALN	**0.98**	0.14
EB	**1.08**	**0.74**
SS	**1.95**	0.05
GC	**1.39**	**0.52**
MST	**0.48**	0.06
GE	**0.79**	**1.77**
MAV	**2.04**	**0.36**
CM	0.01	**0.20**
ESN	**0.30**	**0.47**
CMR	0.06	**0.46**
MHU	0.02	0.03
SVE	**1.99**	**0.58**
MMO	**0.17**	**0.42**
MSA	0.06	0.06
MAQ	**0.38**	**0.17**
AW	0.01	0.03
NLA	0.03	0.02
recognition frequency	14/20	13/20

C

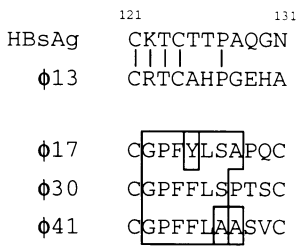

Fig. 1. Identification of disease-specific phagotopes. Binding of the selected phagotopes to antibodies present in human sera was detected by ELISA on immobilized phage. (**A**) Reactivity of selected phagotopes with negative sera. For each serum (first column), binding of antibodies to the tested phagotope and to wild-type phage was detected. Average values ($A_{405\ nm}$) from two independent experiments have been determined. Results are expressed as the difference between the average value of the tested phagotope and that of wild-type phage. Numbers in bold indicate statistically significant reactivities. The highest standard deviation (σ_{max}) observed for all groups of data was 0.05. We considered values as statistically significant when differing more than $3\sigma_{max}$ ($P < 0.003$) from the background signal observed for the wild-type phage. The frequency of recognition of each phagotope by sera is indicated. (**B**) Reactivity of phagotopes ϕ13 and ϕ41 with positive sera. (**C**) Peptide sequences of disease-specific mimotopes. The predicted amino acid sequences of peptides expressed by clones ϕ13, ϕ17, ϕ30 and ϕ41 are reported. The partial sequence of HBsAg and its homology with clone ϕ13 are also reported. Numbers refer to the amino acid positions of the HBsAg peptide, as predicted from the nucleotide sequence of the S gene of the hepatitis B virus genome (*adw* subtype).

phage were used as positive and negative controls, respectively. Recognition of HBsAg by antibodies present in the sera of mice immunized with ϕ13 and ϕ41 was completely inhibited by the HBsAg itself, and by the relative immunizing phage, but not by wild-type phage (Figure 3C and D). These data prove that the serum-selected phage are both antigenic and immunogenic mimics of the natural antigen HBsAg.

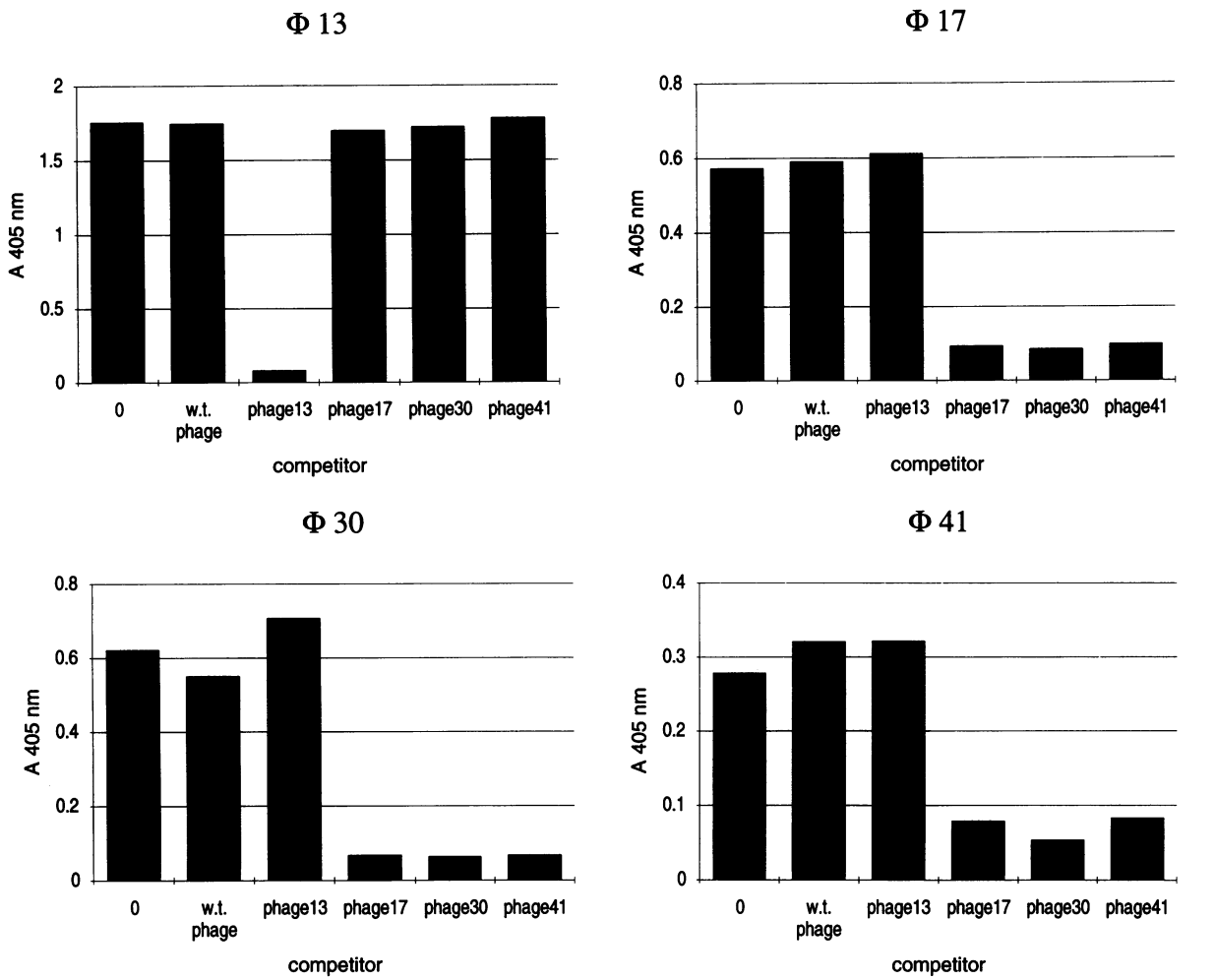

Fig. 2. Serum-selected phagotopes represent two different epitopes. Inhibition of phagotope recognition by antibodies present in the CB serum measured in ELISA. Coated phagotopes are indicated at the top of each panel. Competitor phage are indicated at the bottom. Average values from two independent experiments are reported.

Discussion

In the past, affinity selection from RPL using homogeneous ligates such as mAbs has revealed novel ligands which mimic the binding properties of the natural antigen. In this work, we have explored the possibility of using human sera to identify and characterize mimotopes from RPL. Our goal was the development of a general strategy for the discovery of disease-related epitopes also in those cases were the antigen is not known or it is not available.

Our attempts to directly subtract from the library those phage reacting with antibodies normally present in the sera of healthy individuals have been unsuccessful, presumably due to a low efficiency of the selection procedure. By affinity selection and immunological screening of plaques on filters with two different sera from immunized individuals, we selected phage specifically reacting with serum antibodies common to both sera. Among these we have identified phage bearing HBsAg peptide mimics by an 'antigen-independent' procedure which makes use only of sera from patients and normal individuals. The importance of screening the serum-enriched library with an immune serum different from the one used for the selection is 2-fold. First, it allows the identification of phagotopes among which the percentage of disease-specific ones is significantly higher than that observed when the same serum is used for both the enriching and the screening steps (13% versus 1%; Figure 1A and data not shown). Second, in the light of their use as diagnostic reagents and immunogens, the use of two different immune sera should decrease the chance of selecting idiosyncratic epitopes. In principle, it should be possible to identify a core of clones reacting only with antibodies common to a selected population by iterative screening of serum-enriched phage with many immune sera.

The possibility of using serum-selected phagotopes as diagnostic reagents depends on the frequency with which they are recognized by different patient sera. In our model system, 80% of the tested immunized population was scored positive using just two disease-specific phagotopes (Figure 1B). Since we characterized only a very small portion of the phage that reacted with the enriching and screening positive sera (~1%), it is likely that more disease-specific mimotopes would be identified by analysing larger numbers of phage. Such an extensive approach should provide a set of reagents sufficiently 'complete' to allow patient sera to be screened with a high diagnostic sensitivity. Experiments presently ongoing in our laboratory indicate that

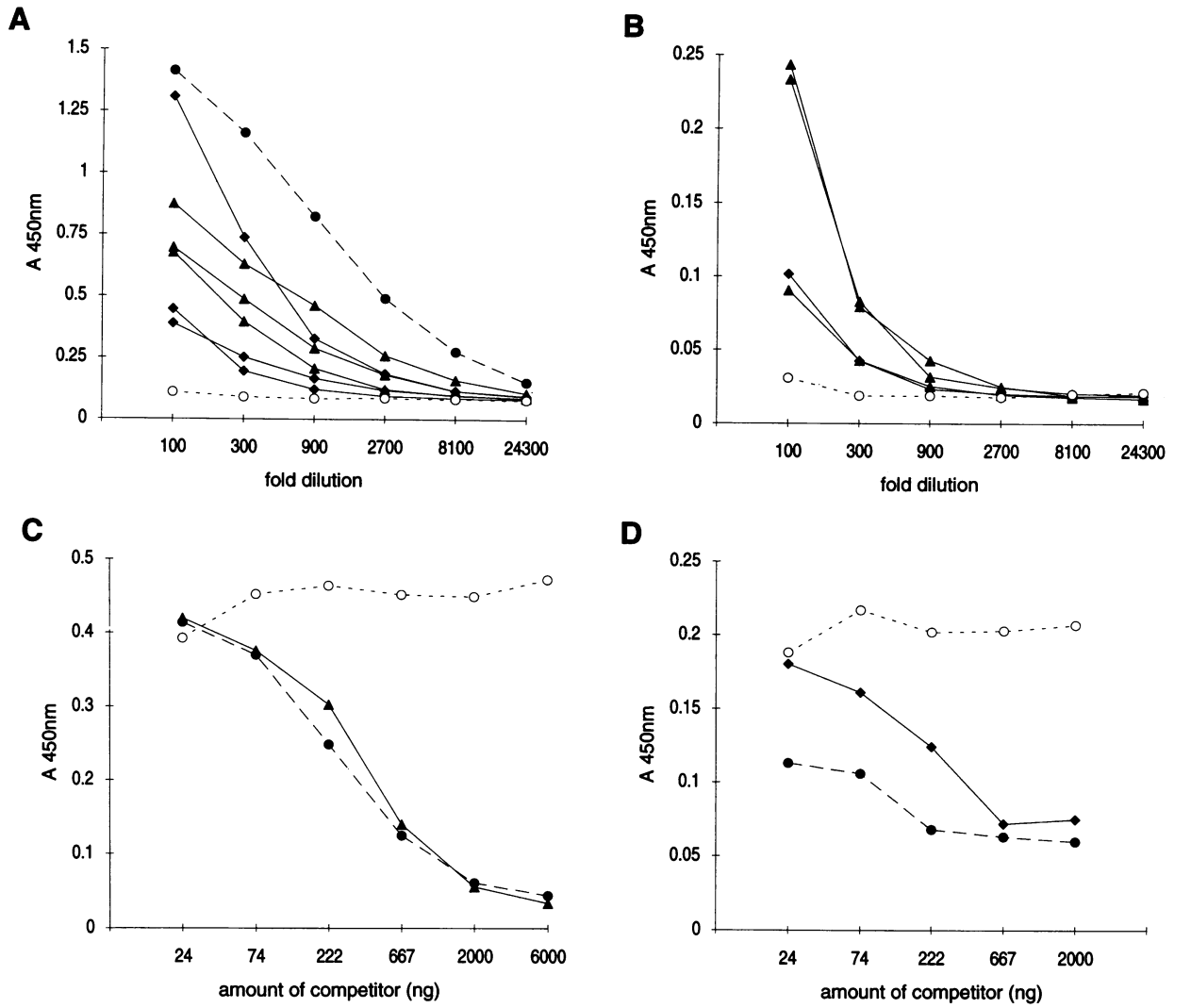

Fig. 3. Phagotopes ϕ13 and ϕ41 are HBsAg immunogenic mimics. (**A**, **B**) Reactivity to HBsAg of sera from mice immunized with ϕ13 (A) and ϕ41 (B) was assayed in ELISA. Tested dilutions are indicated on the abscissa. Average values from two independent experiments are reported. Serum from mouse immunized with HBsAg (●); serum from mouse immunized with wild-type phage (○); sera from mice immunized with phagotopes (◆, BALB/C; ▲, C57Bl). (**C**, **D**) Competition in ELISA of the binding to HBsAg of sera from mice immunized with ϕ13 (C) and ϕ41 (D). Serum from a C57Bl mouse immunized with ϕ13 was diluted 1/300 (C); serum from a BALB/C mouse injected with ϕ41 was diluted 1/100 (D). Amounts of competitor are reported on the abscissa: wild-type phage (○), HBsAg (●), ϕ13 (▲) and ϕ41 (◆). Average values from two independent experiments are reported.

this is also feasible in systems other than that of HBsAg immunization (C.Prezzi and P.Monaci, unpublished data). In addition, the selected disease-specific mimotopes might be improved by *in vitro* maturation to obtain a more accurate and comprehensive reproduction of the natural epitopes.

Contradictory results have been previously reported as to whether epitope mimics selected with mAbs from RPL can elicit antibodies able to recognize the antigen against which the mAb was originally raised (Felici *et al.*, 1993; Keller *et al.*, 1993; Motti *et al.*, 1994). However, a mAb represents only a small portion of the immune response to the antigen. Furthermore, the protocols used for selecting mimotopes with mAbs tend to drive selection toward structures which show the 'best fit' to the ligate, and may not actually correspond to efficient immunogens. In our model system, both serum-identified mimotopes were able to induce a strong and specific response against the natural antigen in mice. Immunization of other animals (rats and rabbits) produced similar results, thus indicating that the immunogenic potential of the selected phagotopes is not species specific (A.Meola and G.Galfré, in preparation). In view of the difference between mouse, rabbit and human antibodies, the finding that phagotopes selected with human sera can raise an immune response in the other two species lends further support to the conclusion that the selected peptides really do mimic parts of the natural antigen HBsAg.

The disease-specific mimotopes were also produced as synthetic peptides, as fusions to the minor coat protein pIII displayed on the surface of the phage and as N-terminal fusions to the multimeric human H-ferritin produced in bacteria (Levi *et al.*, 1987). From a comparative analysis of the response induced by the selected mimotopes in all these molecular environments, it appears that the most immunogenic form is as a pVIII fusion in the context of the original chimeric phage (A.Meola and G.Galfré, in preparation). Grafting previously known immunogenic

epitopes onto phage coat proteins and using them as immunogens has already been reported (de la Cruz *et al.*, 1988; Greenwood *et al.*, 1991; Minenkova *et al.*, 1993; Willis *et al.*, 1993). Our data show that phagotopes, selected exclusively on the grounds of their binding to disease-specific serum antibodies, can act as efficient immunogenic mimics. Whether these serum-identified phagotopes can induce a protective response in a primate model of infection remains to be seen. In any case, the present work could provide key technology for a simple and inexpensive route to vaccine development, which can be applied to diseases whose aetiological agent is unknown.

Our knowledge of the complexity and diversity of the humoral immune response to the same aetiological agent among different individuals is still fairly poor. Nonetheless, studies carried out on pathological states, such as infectious, allergic and autoimmune disease (Cohen and Young, 1991), have suggested that a similar repertoire of antibody genes is expressed by unrelated individuals affected by the same disease. We view the combined use of human sera and phage display technology as a novel instrument to dissect at very high resolution these common features, i.e. the immunofootprint of the aetiological agent. This approach could make footprinting the immune response against a given antigen possible, even in the absence of any information on the aetiological agent. To achieve this, the only requirement is for clinically well-characterized sera from patients and normal individuals. Information gathered through this methodology could greatly improve the diagnosis and prognosis of diseases, and provide reagents useful for the identification of unknown aetiological agents.

Materials and methods

Affinity selection

The human sera used in this study have been tested for the presence of anti-HBsAg antibodies (AUSAB® EIA test; Abbot Labs, Chicago, IL). Sera were precipitated with $(NH_4)_2SO_4$ (40%), resuspended in the original volume of phosphate-buffered saline (PBS) and dialysed twice against 1000-fold their volume of PBS for 2 h at 4°C. Polystyrene beads (6.4 mm diameter; Precision Plastic Ball Company, Chicago, IL) were coated with $(NH_4)_2SO_4$-precipitated sera at a concentration of 10 μg of antibody/ml in coating buffer (50 mM $NaHCO_3$, pH 9.6) for 12 h at 4°C on a rotating wheel. In our experimental conditions, ~1 μg of immunoglobulins was bound per bead. After washing with PBS, beads were blocked with 10 mg/ml bovine serum albumin (BSA) in TTBS [0.5% Tween 20, 50 mM Tris–HCl (pH 7.6), 150 mM NaCl] for 4 h at room temperature. Twelve serum-coated beads were pre-incubated with an excess of M13K07-UV killed phage (2×10^{13} phage particles) in 3.4 ml of TTBS containing 1 mg/ml BSA for 4 h at 4°C. Phage (2×10^{11}) were then added to the above pre-incubation mixture and incubated for 12 h at 4°C. Unbound phage were removed and the beads were washed extensively with TTBS at 4°C. Bound phage were eluted by incubating the beads with 2.4 ml of elution buffer (0.1 M HCl adjusted to pH 2.2 with glycine, 1 mg/ml BSA) for 5 min with gentle agitation at room temperature. The eluate was transferred to a polypropylene tube and neutralized with 240 μl of 2 M Tris–HCl (pH 9.0). Unbound and eluted phage were titrated by infection of XL1-blue bacteria. The percentage of clones containing a productive insert was determined by plating infected bacteria on X-Gal/IPTG indicator plates (Felici *et al.*, 1991).

Immunological screening

Plating of serum-selected chimeric phage as p.f.u. was performed as follows. XL1-blue bacteria were inoculated into Terrific broth (TB) containing 20 μg/ml tetracycline from a fresh plate of minimal medium containing 20 μg/ml tetracycline. Bacteria were grown with vigorous shaking at 37°C up to an $OD_{600\ nm}$ of 0.18 measured on a 1/10 dilution of the culture in TB (corresponding to $\sim 5 \times 10^8$ cells/ml). Cells were kept incubating for a further 15 min at 37°C with gentle agitation and were used for infection within 60 min (XL1-blue TB cells). XL1-blue TB cells were infected with eluted phage from affinity selection at a multiplicity of infection (m.o.i.) of 10^{-3} and plated onto L-agar, 100 μg/ml ampicillin, 1% glucose. After incubation at 37°C for 12–16 h, bacteria were scraped and collected in Luria broth (LB), 100 μg/ml ampicillin, 10% glycerol and inoculated in LB containing 100 μg/ml ampicillin at an $OD_{600\ nm}$ of 0.05. The culture was grown up to an $OD_{600\ nm}$ of 0.2 and super-infected with M13K07 helper phage at a m.o.i. of 20–50. Ater incubation at 37°C for 15 min, infected bacteria were centrifuged for 5 min at 12 000 r.p.m., the supernatant was discarded and the pellet was washed three times with LB to eliminate non-adsorbed helper phage. About 1×10^5 bacteria were plated on a lawn of XL1-blue cells, plates were layered with nitrocellulose filters and incubated overnight at 37°C. Filters were blocked with immunoscreening buffer (5% non-fat dry milk, 0.1% Nonidet P40 in PBS) for 2 h at room temperature. Crude serum was diluted 1/50–1/100 in immunoscreening buffer containing 10 μl/ml of a lysate from XL1-blue cells and 2×10^9 M13KO7 phage particles/ml. The serum mixture was pre-incubated for 2 h at room temperature, then added to blocked filters. Filters were incubated with the serum overnight at 4°C. After washing with 0.1% Nonidet P40/PBS (washing buffer), anti-human (Fc-specific) alkaline phosphatase-conjugated antibodies (Sigma), diluted 1/5000 in 15 ml of immunoscreening buffer, were added to the filters and incubated for 4 h at 4°C. Filters were washed and developed. XL1-blue extract was prepared from an overnight culture of XL1-blue in TB + 20 μg/ml tetracyline. Cells were centrifuged, resuspended in 1/100 of the original volume of PBS and broken by French press lysis.

Immunoscreening experiments with mouse mAbs (RFHBS6, provided by R.Ellis, Merck, Rahway, NJ, and BA1, provided by A.Longobardi, Radim, Rome) as probes were performed with the same procedure. mAbs were used at a final concentration of 1 μg/ml in immunoscreening buffer without M13K07 phage and XL1-blue extract as carriers. Anti-mouse (Fc-specific) alkaline phosphatase-conjugated antibodies (Sigma) were used at a 1/5000 dilution as secondary antibodies.

ELISA assays

The ELISA assay using phage supernatant and crude human sera will be described (L.Dente, G.Cesareni, G.Micheli, F.Felici, A.Folgori, A.Luzzago, P.Monaci, A.Nicosia and P.DelMastro, submitted).

For cross-inhibition assays between different phage in ELISA, multiwell plates were coated with 2×10^{10} CsCl-purified phage particles/well, washed with 0.05% Tween 20/PBS (washing buffer) and blocked with 5% non-fat dry milk, 0.05% Tween 20/PBS (blocking buffer). Human sera were diluted 1/40 in blocking buffer containing 1×10^{12} M13 phage particles/ml, 50 μl/ml of XL1-blue lysate and 2×10^{11} particles/ml of CsCl-purified inhibitor phagotope. The mixtures were pre-incubated overnight at 4°C and then incubated with phage-coated plates for 3 h at 4°C. For control reactions, the inhibitor phagotope was omitted. After washing, anti-human (Fc-specific) alkaline phosphatase-conjugated antibodies (Sigma) were added and incubated at 4°C for 2 h. Plates were washed and developed.

For ELISA assays with mouse sera on HBsAg-coated plates, 100 μl/well of a 5 μg/ml solution of purified yeast recombinant HBsAg (*adw*; obtained from R.W.Ellis, Merck, Rahway, NJ) were used to coat multiwell plates. After washing and blocking, plates were incubated with mouse antisera, diluted as indicated in the text in PBS/0.8% BSA. Incubation was performed at room temperature for 2 h. Plates were then washed and incubated with peroxidase-labelled rabbit anti-mouse antiserum (Dako) for 2 h at room temperature. After washing, plates were washed and developed. For competition assays, CsCl-purified phage, or purified recombinant HBsAg, were added in the indicated amounts to the serum incubation mixtures at the moment of dispensing into the wells.

Mice immunization

Immunizing phage were prepared from XL1-blue infected cells and CsCl purified as previously described. Three- to five-week-old female C57Bl and BALB/C mice (Charles River) were immunized by i.p. injection of 100 μl of antigen solution at day 0, 21 and 42, and bled at day 52 (third bleed) and day 148 (fourth bleed). Phage were injected as 0.9% NaCl suspensions at a concentration of ~0.3 mg/ml (2.5×10^{13} phage particles/ml) without added adjuvant.

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