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Monoclonal antibodies that recognise filamentous phage: tools for phage display technology

(Peptide libraries; phage morphogenesis; phage ELISA; mimotopes; epitope; immunology)

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SUMMARY

We generated six hybridoma cell lines that secrete monoclonal antibodies (mAb) which specifically bind filamentous phage coat proteins. Two of these mAb recognise epitopes that include the N terminus of the coat protein III (pIII), while two others are specific for the N terminus of the major coat protein VIII (pVIII). These mAb are valuable tools to study phage assembly and structure. Furthermore, we describe two examples of how these mAb can be exploited in the construction and screening of peptide libraries displayed by the filamentous phage major coat protein. We have used one of these mAb to develop a sensitive ELISA with crude phage supernatants. This assay allows rapid screening of large numbers of clones from random peptide phage libraries. Some of the anti-phage mAb described here can interfere with wild-type phage propagation, while phage carrying modifications in their coat proteins are insensitive to growth inhibition. We have exploited this observation as a tool to favour the growth of phage displaying peptides fused to pVIII, with respect to vector phage.

INTRODUCTION

Display of peptides on the surface of filamentous phage by fusion to the N terminus of pIII or pVIII is a powerful tool for the identification and/or characterisation of ligands for many different target molecules (Ab, receptors or other proteins; for reviews, see Cesareni, 1992; Smith and Scott, 1993). Using a variety of display methods, vast libraries of short peptides of random sequences have also been constructed (Scott and Smith, 1990; Cwirla et al.,

1990; Devlin et al., 1990; Felici et al., 1991; Luzzago et al., 1993). Affinity selection of such libraries, using monoclonal and polyclonal antibodies, has revealed novel ligands (mimotopes) which mimic the binding properties of the natural ligand.

Several different techniques for the identification and/or characterisation of positive clones have been described (Smith and Scott, 1993). However, none of these procedures is suitable for rapid and sensitive analysis of large numbers of clones. Here we describe the generation

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Abbreviations: A, absorbance (1 cm); aa, amino acid(s); Ab, antibody(ies); Δ, deletion; DMSO, dimethylsulfoxide; E.M., electron microscope(ic); ELISA, enzyme-linked immunosorbent assay; Fc, constant fragment of IgG; HbSAg, human hepatitis B surface antigen; IgG, immunoglobulin G; mAb, monoclonal Ab; pIII, minor coat protein

of filamentous phage; pVIII, major coat protein of filamentous phage; PBS, phosphate-buffered saline (2 mM NaH₂PO₄/16 mM Na₂HPO₄/150 mM NaCl, pH 7.5); PEG, poly(ethylene glycol); pfa, plaque-forming ability; pfu, plaque-forming unit(s); TB, terrific broth [(per liter) 12 g Bacto-tryptone/24 g Bacto-yeast extract/4 ml glycerol/17 mM KH₂PO₄/72 mM K₂HPO₄]; TBS, tris-buffered saline (50 mM Tris-HCl pH 7.5/150 mM NaCl); TU, transducing unit(s); [] denotes plasmid-carrier state; wt, wild type.

and the characterization of a set of rat mAb that recognise phage coat proteins. These Ab represent useful tools for investigating phage assembly and structure. In addition, we show that these mAb can be used for improving selection and screening of positive clones from peptide libraries displayed on phage.

RESULTS AND DISCUSSION

(a) Generation and characterization of anti-phage mAb

To generate mAb against filamentous phage proteins, a suspension of M13 phage was used to immunize rats and to identify the hybridoma cell lines producing anti-phage antibodies. Six hybridoma supernatants were identified and further characterized by class determination (Table I). Since the anti-M13 Ab secreted by the selected

TABLE I
Characterization of anti-filamentous phage rat mAb

Rat mAb YOPH1 ^a	Idiotypic ^b	pIII-test ELISA ^c	Inhibition assay ^d
51-F9	IgGγ2B	—	++
57-D1	IgGγ1	+	+
48-E10B7	IgM	—	—
44-F2	IgG	+	—
47-H12	IgG	—	+++
7-C4	IgM	—	—

^a Rats (AO and Lou female, 5 weeks old) were immunized intraperitoneally 3 times at three-week intervals with CsCl-purified M13 wt phage and they were killed at the 10th day post-injection. Rat myeloma YO, growing in roller, were used as parent line in somatic cell fusions. Primary screening of the hybrids growing wells was performed using indirect ELISA assay. Microtitre plates (Immuno plate maxisorp, Nunc) were coated overnight at room temperature with the same phage preparation as used for the immunization (5×10^{10} pfu/well).

^b Class determination was done by Ouchterlony double-immunodiffusion assay (Ouchterlony et al., 1986) and also by 0.1% SDS-10% polyacrylamide gel electrophoresis analysis of the [¹⁴C]lysine metabolically-labelled mAb.

^c **ELISA:** Microtitre plates (Immuno plate maxisorp, Nunc) were coated for 12 h at room temperature with purified recombinant pIII (1 µg/well) produced by plasmid pB360-2A (kindly supplied by Dr P. Keller, Merck, West Point, PA, USA), blocked with 10% fetal calf serum in PBS for at least 2 h at room temperature. Ab binding to the antigen was performed for 2 h at room temperature and after several washes with the same buffer, the antigen/Ab complex was evidenced with a peroxidase conjugated rabbit anti-rat Ig (Dako, Denmark), and the substrate used was TMB (Tetramethylbenzidine, Sigma T8768) (Martin et al., 1984).

^d Ab inhibition of phage growth was tested by titration of phage suspensions with and without Ab. **Methods:** Aliquots of 4 µl of serial dilutions of f1 suspension were applied onto a solid growth medium containing 4×10^6 bacteria/ml and 20 µl of supernatant of each hybridoma culture. After 12 h of incubation at 37 °C the phage titre was compared to that obtained onto a control plate without Ab. Number of (+) indicates the efficacy of inhibition.

hybridomas bound the related phage f1 with comparable strength, either of these phage was used in subsequent experiments.

Two of the selected mAb (44-F2 and 57-D1) recognised a purified recombinant pIII in ELISA (Table I). In order to identify the target of the other Ab, we used gold-conjugated anti-rat IgG secondary Ab to decorate phage/mAb complexes for electron microscope (E.M.) observation. In Fig. 1, the decoration patterns obtained with the four Ab of the IgG class are shown: 47-H12 and 51-F9 Ab are detected along the entire surface of the phage capsid (Fig. 1a,b), suggesting that their target is the major coat protein. 57-D1 is always detected at one extremity of the phage (Fig. 1c), thus confirming the ELISA results (Table I), which indicated that this Ab recognizes the pIII protein. By contrast, a very low signal, if any, is detected on the grid that contains 44-F2 (Fig. 1d).

(b) Inhibition of phage propagation

Some antiviral Ab interfere with the virus life cycle. Since this could provide a tool to map the aa on the capsid proteins that are responsible for the interaction with the Ab, we decided to explore the effect of our mAb on phage propagation. This was done by a simple inhibition assay, where hybridoma supernatants were tested for their capacity to inhibit the plaque-forming ability (pfa) of serial dilutions of a phage suspension. This analysis indicated that 57-D1, 51-F9 and 47-H12 inhibit phage propagation. In contrast, the presence of 44-F2, 48-E10B7 and 7-C4 in the plating mixture did not affect phage titre (Table I). To better quantitate the extent of inhibition, as a function of Ab concentration, we used partially purified mAb, obtained by 50% (NH₄)₂SO₄ precipitation of the hybridoma supernatants. In Fig. 2 the results of the inhibition assay performed with different amounts of (NH₄)₂SO₄-precipitated 44-F2, 51-F9, 57-D1 and 47-H12 mAb are shown. The results of this experiment confirmed the data obtained with culture supernatants: mAb 51-F9, 57-D1 and 47-H12 interfered with phage growth, while mAb 44-F2 did not, even at high concentration (40 µg/ml). This is in agreement with the E.M. observations and suggests that mAb 44-F2 has a very low affinity for pIII on phage. MAb 47-H12 is the most effective of our inactivating Ab: it completely abolished phage growth at a concentration of 8 µg/ml. Finally, mAb 51-F9 and 57-D1 displayed intermediate inhibition abilities.

(c) Epitope mapping

To identify specific aa residues involved in mAb recognition, we selected mutants that are insensitive to the inactivating Ab and can form plaques in the presence of

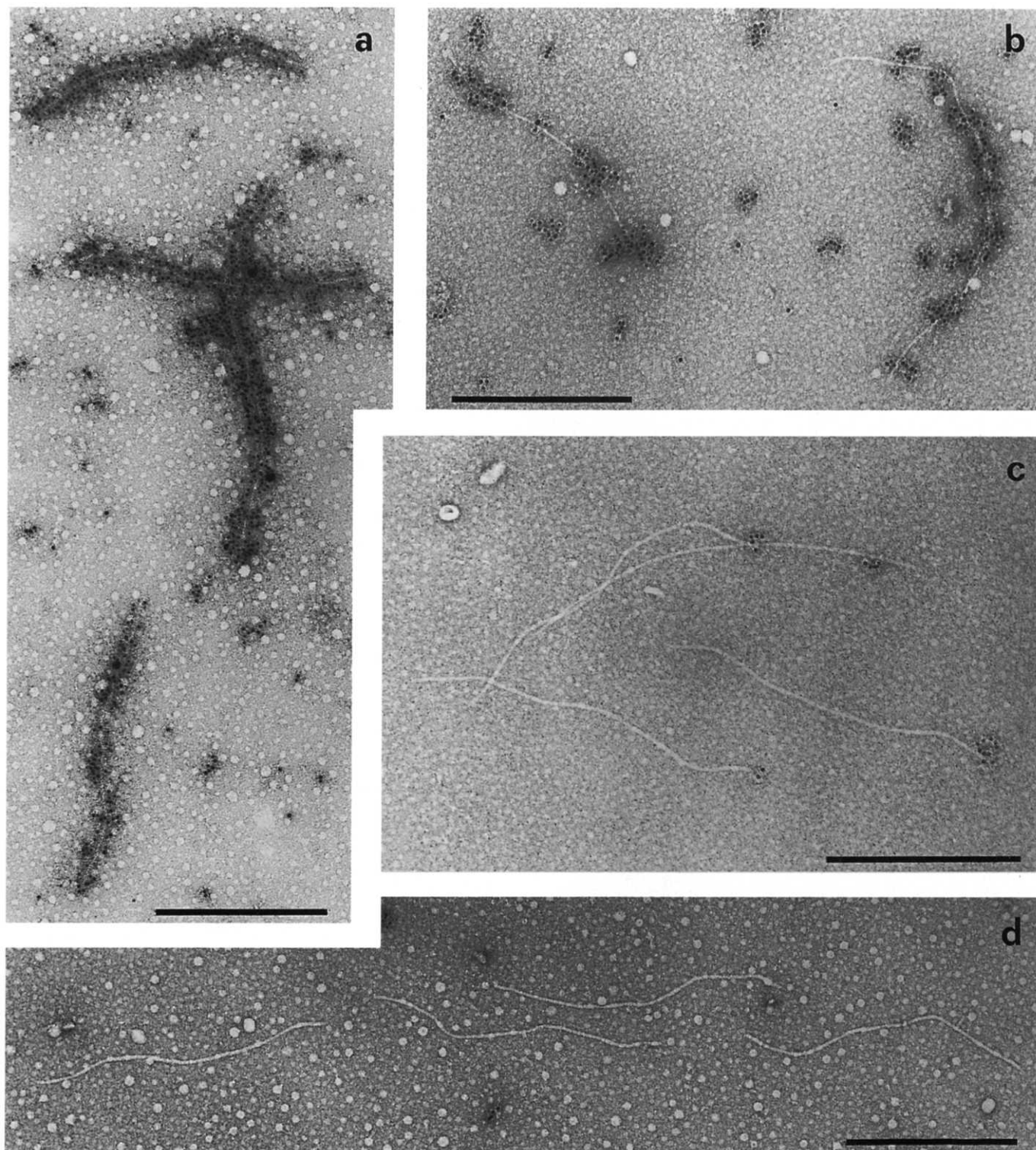


Fig. 1. Electron micrographs of f1/Ab complexes: 47-H12 (a); 51-F9 (b); 57-D1 (c); 44-F2 (d). Bar represents 0.5 μm . **Methods:** f1 phage particles were purified by CsCl gradient and subjected to reduction with NaBH_4 , as described by Lopez and Webster (1983). Samples were incubated at 70°C for 10 min and then adsorbed at room temperature onto carbon-coated 400 mesh nickel grids for 10 min. All successive steps were carried out at room temperature. After a brief rinse in TBS/0.1% BSA, the grids were floated for 30 min on 10 μl -drops of $(\text{NH}_4)_2\text{SO}_4$ purified mAb preparations, diluted in TBS/0.1% BSA to 1.6 mg per ml (51-F9); 1 mg per ml (44-F2); 0.8 mg per ml (57-D1); 1.25 mg per ml (47-H12). After washing for 10 min in TBS/0.1% BSA and washing three times for 5 min in TBS, the grids were floated for 30 min on 10- μl drops of the secondary Ab (Anti-Rat IgG gold-conjugate, 10 nm, Sigma) diluted 1:25 in TBS/0.1% BSA. After washing as described above, the grids were negatively stained with 2% uranyl acetate/2.5% DMSO.

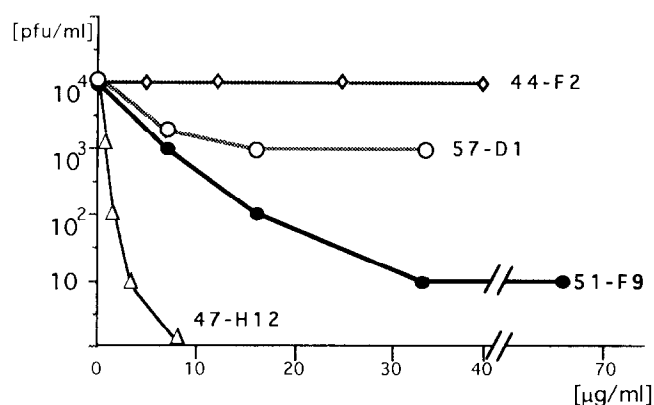


Fig. 2. Plaque-forming ability of phage f1 at different concentrations of mAb. Inhibition assay was performed as described in Table I, footnote d, using different amounts of (NH₄)₂SO₄-purified mAb.

inhibitory concentrations of mAb. One mutant from each selection was plaque purified in the presence of the Ab. Guided by the results of the E.M. experiments, we sequenced gene *VIII* in the mutants selected with mAb 51-F9 and 47-H12 and gene *III* in the mutant selected with mAb 57-D1. In Table II the aa sequences of the N-termini of the pIII and pVIII proteins in the Ab-resistant mutant phage are listed. The aa substitutions observed in the coat proteins of the mutant phage confirmed the E.M. observations and allowed us to localize the epitope recognized by the mAb in the N-terminal portion of the proteins. In mutant 11.1, selected for its ability to form plaques in the presence of mAb 57-D1, the Glu⁵ of pIII is changed into a Gly; in mutant 7.7, resistant to mAb 51-F9, the Asp⁴ of pVIII is substituted by a Gly; finally, in mutant 12.1, insensitive to mAb 47-H12, the Asp⁵ of pVIII is converted into Ala. This last mutation confers resistance not only to the Ab used in

the selection (47-H12), but also to the other Ab specific to pVIII (51-F9). By contrast, the Asp⁴ to Gly substitution in mutant 7.7 confers resistance only to one of the two mAb.

In conclusion, mAb 57-D1 recognizes an epitope that includes the N terminus of pIII, while mAb 47-H12 and 51-F9 both recognize the N terminus of pVIII, although with somewhat different specificities. More extensive mutagenesis experiments are in progress to evaluate the extension and the exact borders of the two epitopes.

(d) mAb 47-H12 can discriminate between a peptide displaying phage and its parent vector

Peptide libraries that exploit fusion to the major coat protein pVIII have been constructed by utilizing a phagemid system that permits the display of few copies of hybrid coat protein dispersed in a otherwise wt phage capsid (Felici et al., 1991). In fact, it was initially observed that at least some peptides that are longer than 6 aa are hardly tolerated since they probably interfere with phage assembly (Greenwood et al., 1990; Felici et al., 1991). However, we have recently found that this finding is not general and that many peptides as long as 10 aa are tolerated by the N terminus of pVIII (G. Iannolo, O. Minenkova and G.C., unpublished). This has permitted the construction of relatively large collections of phage where the entire phage filament is made of hybrid coat proteins.

In these libraries however, as in most phage libraries, several phage carrying a peptide insertion grow less vigorously than the contaminating parent vector. This is a common problem encountered in panning experiments where the enrichment factor gained by a good 'binder'

TABLE II

Single point mutants, selected for the ability to grow in the presence of mAb

Phage ^a	mAb ^b	Growth inhibition ^c			pVIII sequence ^d	pIII sequence ^d
		57-D1	51-F9	47-H12		
f1	none	+	+	+	GCT GAG GGT GAC GAT A ¹ E ² G ³ D ⁴ D ⁵	GCT GAA ACT GTT GAA A ¹ E ² T ³ V ⁴ E ⁵
11.1	57-D1	—	+	+	GCT GAG GGT GAC GAT A ¹ E ² G ³ D ⁴ D ⁵	GCT GAA ACT GTT GGA A ¹ E ² T ³ V ⁴ G⁵
7.7	51-F9	+	—	+	GCT GAG GGT GGC GAT A ¹ E ² G ³ G⁴ D ⁵	GCT GAA ACT GTT GAA A ¹ E ² T ³ V ⁴ E ⁵
12.1	47H12	+	—	—	GCT GAG GGT GAC GCT A ¹ E ² G ³ D ⁴ A ⁵	GCT GAA ACT GTT GAA A ¹ E ² T ³ V ⁴ E ⁵

^a To increase the frequency of spontaneous mutation we have propagated bacteriophage f1 on a *mutD* derivative of strain 7118 (*Δlac-pro*) *thi* [*F' lacI^q lacZΔM15 proAB*']*supE* (Messing et al., 1977).

^b mAb used for selecting Ab-insensitive mutant phage.

^c mAb tested in the inhibition assay.

^d Nucleotide sequences were determined by dideoxy termination method using synthetic oligodeoxynucleotides as primers. Mutated nt and aa are in bold.

can be partly or completely lost in the amplification step. To alleviate this difficulty we have explored the possibility of using anti-phage mAb to favour the growth of recombinant over vector phage.

While testing several phage mutants for their sensitivity to the anti-phage mAb, we observed that pM30, a phage vector that we generally utilize for the construction of pVIII-displayed libraries (G. Iannolo, unpublished), is much more sensitive to inhibition by 47-H12 than most of its insertion derivatives. Fig. 3 illustrates a typical experiment, where we have compared the plaque-forming ability of vector pM30 and of a recombinant derivative (B1) at varying concentrations of mAb 47-H12.

At an Ab concentration of 5 µg/ml, pM30 shows a reduction of pfa that is at least 100-fold higher than any of the insertion mutants that we have tested (Table III). However, the clones shown in Table III are inhibited to

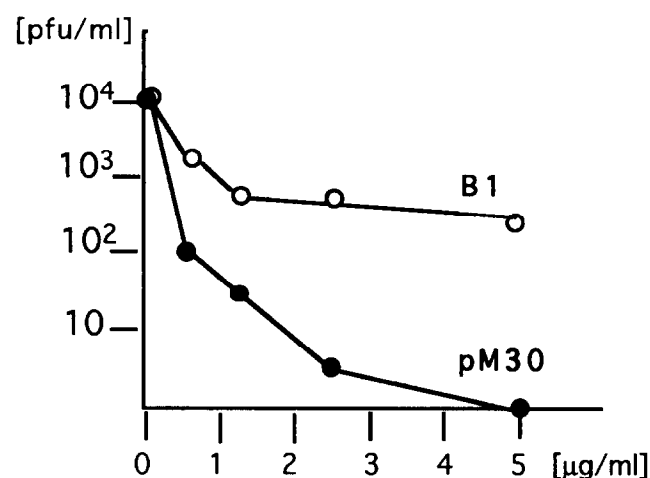


Fig. 3. Plaque forming ability of vector pM30 and of clone B1 at varying concentrations of mAb 47-H12.

TABLE III

Inhibition of pfa by incubation of pM30 and its insertion derivatives with mAb 47-H12

Phage ^a	pVIII N-terminal sequence			Reduction of pfa (-fold)
pM30	AEGEF	Q	DPA	10 000
5	AEGES	RYFLNSAAP	DPA	100
7	AEGEF	HNRSTVDIP	DPA	50
8	AEGEF	RTGYGSAPT	DPA	50
12	AEGEF	YGGGRSSAT	DPA	50
14	AEGEF	TPLHFDSGA	DPA	100
23	AEGEF	THYQGRAGA	DPA	50
B1	AEGEF	AQMIARTAS	DPA	50
B2	AEGEF	AQMSARTAS	DPA	50

^a The vector pM30 derives from f1 phage: it carries a mutagenized gene VIII, containing an *EcoRI* and a *BamHI* restriction site in the region encoding the amino-terminus of the protein. Random oligodeoxynucleotides, inserted between these two sites, generate hybrid pVIII that display the corresponding peptides on the phage surface.

different degrees. Inspection of the aa sequences of the peptide inserts does not permit a straightforward interpretation of the different sensitivities observed. We have observed however that a judicious choice of the Ab concentration permits to decrease substantially the plaque forming ability of the vector, without interfering with the majority of the insertion clones.

(e) mAb 57.D1 can be exploited to perform rapid ELISA with crude phage supernatants

Construction and screening of peptide libraries based on the highly multivalent pVIII display system has been previously described. The published methods for the identification of positive clones from affinity selected phage pools require multiple experimental steps (phage purification for dot blot and ELISA, or phage titration after micro-panning experiments) which render the screening of large number of clones very cumbersome if not impossible. To overcome this limitation we explored the possibility of using anti-phage mAb to tether phage particles onto the surface of multiwell plates directly from crude supernatants of infected bacteria, without any previous purification step. We reasoned that anti-pIII mAb should link the phage to the plate, leaving peptides displayed as fusion to the major coat protein free to interact with the target Ab. For our experiments we selected mAb 57-D1 for its high affinity to pIII as shown by the infection inhibition assay and by E.M. analysis (Table I, Fig. 1).

The mouse mAb RFHBS-6 which recognises the human hepatitis B surface antigen (HbSAg) was chosen as primary Ab. Phage supernatant of a positive clone (phage 35) previously identified by affinity selection of the pVIII9aa-cys library (Luzzago et al., 1993) with mAb RFHBS-6 (Folgori et al., 1994) was prepared. Phage supernatants of two non related clones and wt-phage (phage 1, phage 2 and pC88) were used as negative controls. Equal amounts of phage particles from each supernatant were added to mAb 57-D1 coated wells. After binding of phage particles, primary Ab was added and its reaction with the phage was revealed by an enzyme-linked anti-mouse secondary Ab. As shown in Fig. 4 the signal observed with phage 35 is 50-fold higher than that obtained with wt-phage. Both non related phage show background levels of reactivity (Fig. 4). These results indicate that phage 35 is specifically recognised by mAb RFHBS-6 in this assay.

Optimal mAb coating conditions, amount of supernatant, time and temperature of each incubation step were determined and are reported in the legend of Fig. 4.

The anti-pIII mAb 57-D1 can also be successfully used in similar ELISA experiments with human mAb as primary Ab (R. Bandi, unpublished). Until now, specific epitope recognition by polyclonal Ab using crude phage

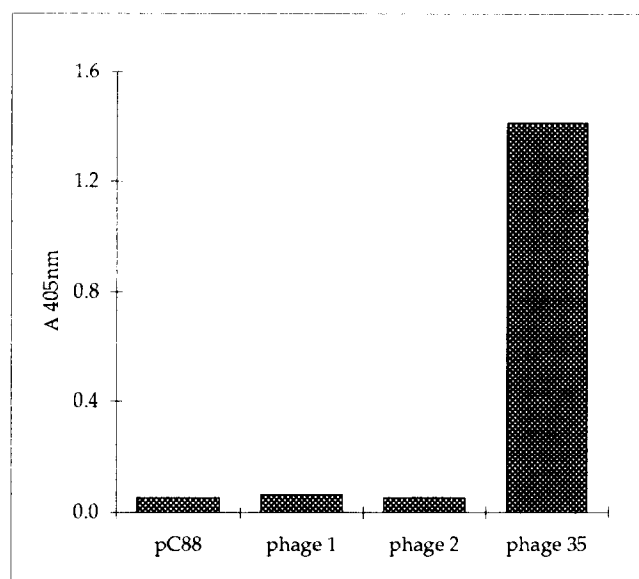


Fig. 4. Reactivity of different phage clones with the mouse mAb RFHBS-6 on ELISA plates coated with mAb 57-D1. Average values ($A_{405\text{ nm}}$) from three independent experiments have been determined. **Methods:** Multiwell plates were coated with $1\text{ }\mu\text{g/ml}$ of $(\text{NH}_4)_2\text{SO}_4$ -precipitated mAb 57-D1 in 50 mM NaHCO_3 , pH 9.6, for 12 h at $4\text{ }^\circ\text{C}$. (The highest signal and the highest signal to noise ratio were observed with mAb coating conditions between 0.5 and $1\text{ }\mu\text{g/ml}$). After washing several times with washing buffer (PBS/0.05% Tween 20), $250\text{ }\mu\text{l/well}$ of blocking buffer (5% non-fat dry milk/0.05% Tween 20/0.02% NaN_3 in PBS) were added and plates were incubated at $37\text{ }^\circ\text{C}$ for 1 h. The blocking solution was then discarded and a mixture of $50\text{ }\mu\text{l}$ of phage supernatant (containing 10^{10} ampicillin transducing units (TU) of phage) and $50\text{ }\mu\text{l}$ of blocking buffer was added to each well. Phage particles were allowed to bind for 1 h at $37\text{ }^\circ\text{C}$. After washing, $100\text{ }\mu\text{l/well}$ of mouse mAb, at a concentration of $1\text{ }\mu\text{g/ml}$ in blocking buffer, was added. Blocking buffer contains 1×10^{11} PEG-purified M13 phage particles, 1×10^{11} PEG-purified pC88 phage particles and $10\text{ }\mu\text{l}$ of crude supernatant from unrelated rat hybridoma cells. The mAb mixture was pre-incubated for 2 h at room temperature prior to addition to phage-coated plates. Incubation was performed at $4\text{ }^\circ\text{C}$ for 12–16 h. Shorter times (2 and 4 h) and higher temperatures ($25\text{ }^\circ\text{C}$ and $37\text{ }^\circ\text{C}$) of mAb incubation were also tested, resulting in lower signals (data not shown). Plates were washed several times with washing buffer and $100\text{ }\mu\text{l/well}$ of anti-mouse (Fc-specific) alkaline-phosphatase conjugated Ab (Sigma), diluted 1:5000 in blocking buffer, were added. After incubation for 4 h at $4\text{ }^\circ\text{C}$, plates were washed several times with washing buffer, one more time with substrate buffer (10% diethanolamine/0.5 mM MgCl_2 /0.05% NaN_3 , adjusted to pH 9.8 with HCl) and developed with $100\text{ }\mu\text{l/well}$ of a 1 mg per ml solution of *p*-nitrophenyl phosphate in substrate buffer for 1 h at $37\text{ }^\circ\text{C}$. The results were recorded as the difference between the absorbance at 405 nm and 655 nm by an automated ELISA reader (Bio-Rad 450).

preparations has been hampered by the high background signal interference, presumably produced by the interaction of serum Ab with bacterial proteins and other contaminants present in the medium. In fact, specific signals from phage coated plates using human sera could be obtained only upon CsCl purification of the phage particles (F.F., unpublished). We therefore tested whether our ELISA system could be used with human sera as

primary Ab. To this end we prepared phage supernatants from six clones (phage 4, phage 8, phage 19, phage 20, phage 24 and phage 41) previously selected for their reaction with Ab present in the serum of an individual immunised against HBsAg ($\sigma+$). One of these clones (phage 41) was shown to be specific for anti-HBsAg Ab present in the sera of many different immunised individuals ((Folgori et al., 1994). The other five clones were recognised by Ab not related to the HBsAg immunisation. All supernatants were tested for their reactivity with the immune ($\sigma+$) and the pre-immune ($\sigma-$) sera from the same individual. The wt-phage containing supernatant was used as a negative control. As shown in Fig. 5, clones phage 4, phage 8, phage 19, phage 20 and phage 24 showed equivalent reactivity with both pre-immune and immune sera, while phage 41 displayed a specific signal only with the immune serum. Both sera displayed a low background signal with wt-phage supernatant. Further analysis with a larger number of sera indicated that the background signal observed with wt-phage is lower than $0.2\text{ } A_{405\text{ nm}}$ for 95% of the tested samples in the conditions described in the legend to Fig. 4 (data not shown).

These results suggest that the described ELISA is a sensitive and reliable system that can be generally used

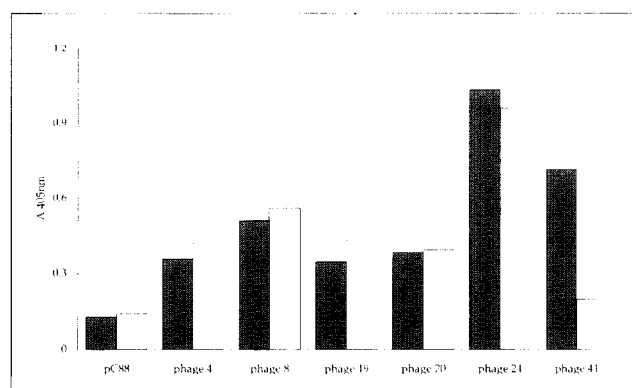


Fig. 5. Reactivity of different phage clones with human sera on ELISA plates coated with mAb 57-D1. Average values ($A_{405\text{ nm}}$) from three independent experiments have been determined. **Methods:** ELISA was performed as described in the legend to Fig. 4, with the following modifications. Human sera were diluted 1:100 in blocking buffer containing 1×10^{11} PEG-purified M13 phage particles, 1×10^{11} PEG-purified pC88 phage particles, $10\text{ }\mu\text{l}$ of crude supernatant from unrelated rat hybridoma cells and $5\text{ }\mu\text{l}$ of XL1-blue extract. The serum mixture was pre-incubated for 2 h at room temperature prior to addition to phage coated plates. Anti-human (Fc-specific) alkaline-phosphatase conjugated Ab (Sigma), diluted 1:5000 in blocking buffer, was used as secondary Ab. XL1-blue (*recA1 endA1 gyrA96 thi hsdR17 (r_k⁺ m_k⁺) supE44 relA1 lac⁻ [F'proAB lacI^q lacZ AM15 Tn10]*) extract was prepared from an overnight culture in TB with $20\text{ }\mu\text{g/ml}$ of tetracycline. Bacteria were centrifuged, resuspended in 1/100 of the original volume of PBS, broken by French Press lysis or sonication and stored in aliquots at $-70\text{ }^\circ\text{C}$. Open and filled bars refer to ELISA experiments with pre-immune and immune sera, respectively.

to screen large numbers of pVIII-displayed peptides with many different homogeneous or heterogeneous ligates.

(f) Conclusions

(1) We have selected six rat hybridoma lines producing Ab that recognise filamentous phage coat proteins. Four of them are of the IgG class and have been characterized in more detail. ELISA with purified pIII protein, electron microscopy and selection of Ab-insensitive mutants indicate that two of these four Ab recognise pIII, while the remaining two specifically bind the N terminus of pVIII. These mAb represent a valuable tool to study virus assembly and structure.

(2) Anti-phage mAb 47-H12 discriminates between vector PM30 and its insertion derivatives. This observation has been exploited to alleviate the background problems that arise in library construction when a vector grows better than its recombinant derivatives.

(3) mAb 57-D1 can be exploited to perform rapid and sensitive ELISA using crude phage supernatants without any intermediate purification steps being necessary.

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