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Antibodies in haystacks: how selection strategy influences the outcome of selection from molecular diversity libraries

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Abstract

Antibodies against most antigens can be isolated from high quality phage antibody libraries. However, not all antibodies binding a particular antigen are necessarily found when standard selections are performed. Here we investigate the effect of two different selection strategies on the isolation of antibodies against a number of different antigens, and find that these different strategies tend to select different antibodies, with little overlap between them. This indicates that the full diversity of these libraries is not tapped by a single selection strategy and that each selection strategy imposes different selective criteria in addition to that of antigen binding. To fully exploit such libraries, therefore, many different selection strategies should probably be employed for each antigen. The use of alternative strategies should be considered when selection apparently fails, or when the number of different antibodies recognizing an antigen needs to be maximised. Furthermore, the microtitre selection strategy developed is likely to prove useful in the application of phage antibody libraries to the human genome project, allowing the high throughput selection of antibodies against multiple antigens simultaneously.

Keywords: Phage antibodies; Selection; Libraries; Molecular diversity

1. Introduction

Phage antibody libraries (Marks et al., 1991; Griffiths et al., 1994; Vaughan et al., 1996; Sheets et al., 1998; de Haard et al., 1999; Sblattero and Bradbury, 2000) have been used to derive antibodies against a wide variety of different antigens. Many different

Abbreviations: scFv, single chain Fv; PCR, polymerase chain reaction; GST, glutathione S transferase; PBS, phosphate-buffered saline

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selection methods have been devised, including biopanning on immobilised antigen coated onto solid supports, columns or BIAcore sensorchips (Griffiths et al., 1994; Malmborg et al., 1996), selection using biotinylated antigen (Hawkins et al., 1992), peptides (Persic et al., 1999), panning on fixed prokaryotic (Bradbury et al., 1993), or mammalian (Mutuberria et al., 1999) cells, tissue culture cells (Cai and Garen, 1995; Siegel et al., 1997; Hoogenboom et al., 1999), fresh cells (Palmer et al., 1997; Figini et al., 1998), subtractive selection using sorting procedures such as FACS (de Kruif et al., 1995) and MACS (Siegel et al., 1997), enrichment on tissue sections or pieces of tissue (Van Ewijk et al., 1997), selection for internalization (Becerril et al., 1999) and in living animals (Johns et al., 2000), as previously reported for peptide phage libraries (Pasqualini and Ruoslahti, 1996). Additionally, biological methods that exploit the infection machinery of the phage to couple antigen/antibody interaction to infection have also been developed (Krebber et al., 1995; Duenas et al., 1996; Malmborg et al., 1997).

In general, antibodies can be selected against most antigens. However, difficulties occasionally arise, and it has been found that by changing the nature of the antigen used for selection, antibodies with the desired specificity can be found. Lu and Sloan (1999), for example, selected two antibodies recognising a phosphorylated form of the transcription factor, E47. One was selected on the phosphorylated protein directly, while the other required alternative rounds of selection on a phosphorylated peptide and the phosphorylated transcription factor. Neither of the antibodies could be isolated by selecting on the phosphorylated peptide alone. Similarly, Hoogenboom (personal communication) was able to isolate antibodies recognising Muc-1 on tumour cells, only by performing alternating selections on Muc-1 peptides and tumour cells. This illustrates one of the problems with selecting from molecular diversity libraries: clones with desired properties are often present, but cannot be found. This is probably caused by the loss of clones with desirable binding characteristics due to deleterious effects on bacterial growth or phage production, or the domination of the selection by unwanted clones that may be non-expressing deletion mutants (de Bruin et al., 1999), clones which recognise either contaminants or unwanted epitopes (Hoogenboom et al., 1999), or polyreactive clones (Bender et al., 1994; Llorente et al., 1999).

Providing the procedure is appropriately designed, selection of antibodies from phage antibody libraries lends itself easily to automation. In a first step in this direction, we have investigated a microtitre-based method, by which it should be possible to select antibodies against 96 different antigens simultaneously. The use of this method results in a number of changes in the selection conditions when compared to the traditional methods (see Table 1). In order to examine the effect of such changes on the nature of the antibodies selected, we selected antibodies against a panel of 11 different antigens using either the microtitre method or the traditional immunotube method. We find that both methods select essentially independent sets of antibodies, with only a few antibodies found in both selection procedures, indicating that the physical parameters imposed in selection can strongly influence the nature of the antibodies selected from a library.

Table 1
Comparison of two different selection methods
The main differences between the two selection methods used are given. The multipin method is novel, and the immunotube method used is based on that described by Marks et al. (1991), with the main differences that bacteria, rather than triethylamine, were used for elution, and that growth between selection rounds was carried out in liquid culture only. These modifications make the two methods more similar.

Variable	Microtitre method	Immunotube method
Amount of antigen used	Low (10–100 ng)	High (100–1000 ng)
Surface area coated with ag	0.5 cm^2	5.0 cm^2
Phage antibody library	Dilute: 2.5×10^{11} phage/ml	Concentrated: 10^{13} phage/ml
Stringency of washing	High: 4×5 min	Less high: 5–20 quick washes
Number of eluted phage	Low (10^{3-6})	High (10^{6-8})
No. of ags simultaneously	Up to 96	No more than five

2. Materials and methods

2.1. Bacterial strains

DH5αF′ (Gibco BRL): F′/endA1 hsdR17 (r_K^- m_K^+) supE44 thi-1 recA1 gyrA (Nal^r) relA1 Δ(lacZYA-argF)U169 deoR (F80dlacD(lacZ)M15).

BS1365: BS591 F′ kan (BS591: recA1 endA1 gyrA96 thi-1 D lacU169 supE44 hsdR17 [lambda imm434 nin5 X1-cre] (Sauer and Henderson, 1988)).

2.2. Phage antibody library

The antibody library used in this paper was a scFv antibody phagemid library based on that described in Sblattero and Bradbury (2000), with the following improvement: the library was preselected on recombinant protein LA, with V genes subsequently recombined, to improve display levels (Lou et al., in preparation), and purified by cesium chloride density centrifugation. This is a naive human scFv library with an estimated diversity of 10^{11}, calculated on the basis of the number of recombination events observed (see Sblattero and Bradbury, 2000 for details) per cell, and extrapolated to the volume used for library growth.

2.3. Antigens

The following antigens, identified by number in the figures and tables, were used for selection and screening: (1) the last 96 aa of a densin 180 homolog containing a PDZ domain (unpublished); (2) aa 1400–1495 of densin 180 containing a PDZ domain (Apperson et al., 1996); (3) aa 443–596 of Yotiao (Lin et al., 1998); (4) the C terminal 2/3 of SNK (Serum iNducible Kinase) (Simmons et al., 1992); (5) the three tandem immunoglobulin domains of RPTP (Receptor Protein Tyrosine Phophatase) sigma (Pulido et al., 1995); (6) the entire DDP (Deafness Dystonia Protein) coding sequence (Jin et al., 1996); (7) the C terminal half of STAM (Signal Transduction Adaptor Molecule) (Takeshita et al., 1996); (8) the three tandem immunoglobulin domains of RPTP sigma; (9) aa 1216–1505 of SPAR (SPA1-like Rapgap; also called E6TP1) (Gao et al., 1999); (10) aa 723–789 of glutamate receptor (sequence common to all AMPA receptors) (Nusser et al., 1998); (11) membrane proximal, enzymatically active phosphatase domain of RPTP sigma. Antigens 1–7 had glutathione S transferase tags; antigen 8 had an alkaline phosphatase tag, antigens 9 and 10 had a trx–his tag, while antigen 11 had a his tag.

2.4. Phage antibody selection on immunotubes

The protocol used for phage antibody selection was similar to that previously described (Marks et al., 1991). Briefly, proteins were coated to the immunotubes (Nunc, Rochester, NY) at 10 μg/ml overnight in carbonate buffer (pH 9.6, 100 mM) at 4°C, blocked in 2% skimmed milk phosphate-buffered saline (MPBS) for 1 h at room temperature (RT) and incubated with the phage antibody library (purified and concentrated by PEG precipitation and CsCl gradient supercentrifigation). 10^{13} phage particles were blocked with 1 ml of MPBS for 1 h and added to the immunotubes. The immunotubes were gently rocked for 1 h and left still for an additional 2 h at RT. The washing steps involved 5 (first round selection) or 20 (second or third round) times with PBS–0.1% Tween-20 and subsequently with PBS. Antigen binders were eluted with 1 ml DH5aF′ grown in 2XTY 1% glucose to an OD_{600} 0.3 and incubated for 30 min at 37°C without shaking. Ten microliters of infected bacteria were plated on 2XTYAG (2XTY containing ampicillin 100 μg/ml and 1% glucose) plates to titer the eluted phages and the remaining bacterial suspension was used for the next cycle of phage growth and selection.

Phages were prepared from the eluted bacteria, by growing them to an OD_{600} 0.5 in 10 ml of 2XTYAG and infecting with M13K07 helper phage at a multiplicity of infection of 20:1. After 30–60 min at 37°C, bacteria were centrifuged, resuspended in 10 ml of 2XTYAK (2XTY containing 100 μg/ml of ampicillin and kanamycin) and grown overnight at 30°C. Phagemid particles were prepared by PEG precipitation, and resuspension in 1 ml of PBS. Fifty microliters of the phage suspension were sampled for polyclonal ELISA and the remaining part (10^{13} phages as average) underwent the next cycle of selection. Polyclonal pools and/or randomly picked

individual clones after each round of selection were tested for their reaction with each protein by ELISA.

2.5. Phage antibody selection on multipins

One hundred microliters of each of the 11 target proteins dissolved in carbonate buffer (pH 9.6, 100 mM) at 10 μg/ml were added to 11 different wells of a 96-well ELISA plate (Nunc). A multipin Nunc-Immuno TSP plate (Nunc, Naperville, IL) was used as a cover for the 96-well plate. This immerses an individual pin into each of the wells, thus coating it with the antigen. Coating was carried out overnight at 4°C. Both the 96-well plate and the TSP pins were blocked using 2% MPBS at room temperature for 1 h. The plate was kept at 4°C for later use in ELISA. The TSP plate was transferred to a tray containing 40 ml of phage antibody library, previously blocked in 2% MPBS and incubated on a rocking platform with gentle shaking for 1 h at room temperature. The same number of phage particles (10^{13}) was used as in the immunotube selection. The TSP plate was washed by immersing the pins in a new tray containing 40 ml PBST and shaking gently for 5 min for the first selection round, and 10 min for subsequent rounds. This was repeated twice with PBST and twice more with PBS. Phage bound to the pins were eluted by incubation with 100 μl DH5αF′ at OD_{600} 0.3 dispensed into individual wells of a 96-well plate for 30 min at 37°C in a 96-well plate to allow infection to occur. One microliter was used to determine the titre of the output phage and ampicillin (to 100 μg/ml) and M13K07 helper phage at a multiplicity of infection of 20:1 were added to each well and incubated at 37°C for 1 h. The plate was then centrifuged at 1600 rpm for 10 min, and the pellet resuspended in 2XTYAK. The 96-well plate was incubated overnight at 30°C shaking at 250 rpm and centrifuged at 1600 rpm for 10 min. Half of the supernatant of each well (50 μl) after overnight rescue was used directly for the next round selection and half for ELISA detection. The second or third round selection with TSP was performed essentially as in the first round, except that the incubation was not in a tray but in a 96-well plate, containing the phage in each well prepared from the previous round of selection and without any concentration or purification. Washing and other steps were the same as for the first round selection. Phages from the polyclonal pool or from individual clones after each round of selection were tested for their reactivity with their correspondence proteins by ELISA.

2.6. Phage ELISA for positive clone identification

The ELISA plates prepared as above were incubated with 50 μl phage (either monoclonal or polyclonal pools) diluted 1:1 with MPBS for 60 min at room temperature. The plate was washed three times with PBST and three times with PBS, and anti-M13-HRP conjugate (Pharmacia) was added to each well as a second antibody. Washing after 60-min incubation was as described above. Development was carried out with TMB used as the substrate for HRP. The OD_{450} value measured after the reaction was stopped by 1 M H_2SO_4. Positive clones were judged as those which gave ELISA signals at least three times the background value.

2.7. Fingerprinting of the positive clones

The V genes of positive monoclones for each protein were amplified by PCR using V gene primers as described (Marks et al., 1991). The amplified V genes were digested with *Bst*NI and run on a 2% agarose gel to identify the different clones.

3. Results

3.1. Increasing selection cycles reduces diversity

In order to determine the effect of the number of selection cycles upon the diversity of the antibodies isolated, 10 antigens were coated on immunotubes and used to select antibodies. Each selection cycle consisted of binding to antigen, washing, elution, reinfection and phage preparation, as described below. For each antigen, 24 clones after the second, and 16 clones after the third round of selection were assessed for their antigen binding capability. The diversity of these positive clones was assessed by PCR and fingerprinting using *Bst*NI, a restriction enzyme cutting commonly within V genes. The percentage of antibodies recognising each antigen was, on the whole, higher after three rounds of selection

Table 2
Diversity changes with selection round
Ten of the eleven antigens in Table 3 were subjected to selection using immunotubes. After the second and third cycles of selection, 16 to 24 different clones were assessed for binding by ELISA. Positive clones were fingerprinted using *Bst*NI. The numbers of tested, positive and different clones for each round are given.

Selection round	Class	Antigen									
		1	2	3	4	5	6	7	8	9	10
2	tested	24	24	24	24	24	24	24	24	24	24
	positive	11	13	9	9	15	10	7	2	15	16
	diverse	4	7	1	4	8	1	1	2	5	12
3	tested	16	16	16	16	16	16	16	16	16	16
	positive	5	10	8	10	10	10	10	10	10	10
	diverse	1	3	1	2	2	1	1	9	3	5

than after two, although there were two exceptions (antigens 1 and 10). However, this increase in positivity was balanced by a reduction in diversity in all but one case (antigen 8), as illustrated in Table 2. In some cases this reduction in diversity was 50% or more (antigens 1, 2, 4, 5, 10), and it was found that essentially all clones found after three selection cycles were contained within the pool of those found after two selection cycles.

3.2. Using a microtitre format for selection

Although selections from phage antibody libraries have traditionally been carried out using immunotubes (Nunc), and the concept of using tubes to perform selections could be carried to the microtitre format by using 96-well plates, it is likely that a selection procedure based on pins would be more effective. This is because the volume available in a single 96-well (100–200 μl) is relatively small compared to the volume of library usually used for a first round selection (10^{13} phage in 1 ml), reducing the effective diversity sampled. Furthermore, by carrying out individual selections in each of 96 wells, large quantities of library would be consumed. Given that antibodies recognising one antigen will not recognise others, this represents a waste of an extremely valuable resource. By using pins, arranged in the microtitre format, and incubating a single library aliquot with all pins, it should be possible to use a single aliquot to select antibodies against 96 different antigens simultaneously. After the first round of selection, during which diversity is drastically reduced and biased towards the antigen used for selection, phage can be eluted either en masse, allowing phage antibodies recognising different antigens to grow together, or they can be eluted into single microtitre wells, thus keeping each selection independent of all its neighbours. Experiments with a number of different recombinant antigens (data not shown) indicated that when phage antibodies were eluted and grown en masse, the selection tended to be biased towards antigens containing epitopes that were in common (e.g. GST tags) rather than unique epitopes characteristic of each individual antigen. This is not surprising, given that such epitopes will be present in higher concentrations.

For this reason, in subsequent experiments, after first round growth and selection, selected phage were eluted into individual wells and all subsequent growth, elution and selection were conducted in individual wells, keeping each antigenic selection independent. This micropin method is different to the standard selection procedure, in that the amounts of antigen used are lower, the volumes used tend to be smaller, and washing is more stringent (a comparison of the two methods is given in Table 1). This provided a good model to compare the effect of different selection methods, rather than forms of antigen, on the outcome of selection. In order to test this, a phage antibody library, based on that described in Sblattero and Bradbury (2000) (see Materials and methods) was selected on 11 different antigens (Table 3a), either using the micropin method or a modified version of the standard immunotube method (Marks et al., 1991). Between them the two methods yielded 124 different antibodies (assessed by PCR fingerprinting), ranging from 1 to 9 for the microtitre method and 2 to 14 for immunotubes, with a mean of 4.9 different antibodies for the microtitre method, 7.5 for the immunotube method, and a total mean of 11.3 different antibodies. When the identities of the antibodies selected with these two methods were compared by PCR fingerprinting, it was found that completely different antibodies were selected in five cases, there was a single shared clone in two cases, two shared clones in three cases and one case in which four (of the total 21 selected antibodies) were shared, with a maximum of 23% shared antibodies. This indicates that for most anti-

Table 3
Antibodies were selected against the antigens indicated using either the immunotube or microtitre selection methods. Positive clones were fingerprinted using *Bst*NI and each different fingerprint pattern identified. Clones recognizing the same antigen from two different selection methods were considered to be identical if their fingerprint pattern was identical. Altogether, 124 different antibodies were selected against 11 different antigens
Antibody selections against two of the antigens were repeated using both selection methods (at a distance of months from the original selection). The number and percentage of identical antibodies are indicated for each antigen and selection method.

(a) Different selection methods select different antibodies

Antigen	No. diff. positive clones—MP	No. diff. positive clones—IT	Total no. of diff. clones	No. identical clones	% identical clones
1	6	7	11	2	18
2	3	10	12	1	8
3	9	3	12	0	0
4	9	7	14	2	14
5	1	10	11	0	0
6	2	5	6	1	17
7	6	3	9	0	0
8	9	12	17	4	23
9	3	9	12	0	0
10	3	14	15	2	13
11	3	2	5	0	0
Total	54	82	124	12	9.7

(b) Identical selection methods select similar antibodies

Antigen	Method	No. diff. positive clones sel. 1	No. diff. positive clones sel. 2	Total no. of diff. clones	No. identical clones	% Identical clones
2	MP	3	3	4	2	50
2	IT	8	7	13	2	13
10	MP	4	3	4	3	75
10	IT	9	7	13	3	23

gens, these two different (but not so radically different) methods are both able to select a wide range of antibodies, and that in most cases, the antibodies selected are different, even though the antigen used is identical. These results are in contrast to independent selections repeated (at a distance of months) using the same method for two antigens (Table 3b), in which up to 75% of antibodies could be consistently reselected, depending upon the antigen and method used.

This microtitre method clearly has the potential to select antibodies against many antigens in a high throughput manner. This was further demonstrated by selecting useful antibodies against 34 further anti-

Table 4
Antigens against which antibodies have been selected using the multipin method

Species	Antigen
Human	Flap endonuclease, Rad51c, Rad52, cyclin D, cdk2, cdc25A, cdc25C, replication protein A, PARP, Ku70/80, tau, rad51, chk1, UBL1, tissue transglutaminase, C5, CD59, TCC (terminal complement complex), CD55 (Decay accelerating factor)
Pyrobaculum	Phosphog1ycerate dehydrogenase, pyruvate ferrodoxin oxoreductase, hydantoin utilization prot, DNA ligase ATP
Miscellaneous	Five HIV antigen peptide loops, wheat α-gliadin, guinea pig transglutaminase, *B. thuringiensis* Cry lAa, bovine ubiquitin, *S. pombe* p22, *V. cholera* zot, rat delta GABA receptor subunit

gens, in addition to those shown in Table 2 (Table 4). With the exception of some peptide antigens against which antibodies have not been selected using any method, the microtitre method was always successful. Those recognising C5 have been shown to have potent biological effects, stemming from their ability to inhibit the recognition site of C5 convertase (Francesco Tedesco, personal communication).

4. Discussion

Large phage antibody libraries contain up to 10 billion different clones. This is reduced to no more than 5 to 20 after selection on a particular antigen (Griffiths et al., 1994; Vaughan et al., 1996; de Haard et al., 1999; Sblattero and Bradbury, 2000). Both theoretical and experimental considerations make it unlikely that these antibodies represent all specific antigen binders present in the original library. If, as has been proposed, a library of 10^{5-6} should have sufficient shape space to recognise all antigens (Perelson and Oster, 1979), one would expect 10,000 to 100,000 different binding clones per antigen from a library of 10^{10}. This figure is clearly dependent upon affinity: the higher the affinity required, the larger a library would have to be, and consequently the lower the number of antigen binding clones that would be present. Similar figures can be obtained from an examination of the mean (and median) of four different antibodies per antigen isolated from a library of 3×10^7 (Marks et al., 1991; Griffiths et al., 1993), suggesting that libraries 1000-fold larger should yield an average of 4000 different binders per antigen.

That such large numbers of different clones have not been isolated suggests that binding ability alone is not sufficient to guarantee selection. Indeed, it has been found that selection outcome can be varied by altering the form of an antigen presented to a library (e.g. peptide or protein (Lu and Sloan, 1999), or peptide and cells (Hoogenboom, personal communication)), indicating that binding elements present in the original library, although able to bind the selection antigen efficiently when isolated and tested individually, are sometimes unable to be selected. This is likely to be due to the fact that selection, although designed to isolate antigen binding antibodies, represents a complex process in which many other factors play an important, and sometimes dominant, role. The success of any individual binding clone in a selection experiment will therefore depend upon the balance between positive and negative selective forces as well as the presence within the selective environment of irrelevant clones having specific or non-specific selective advantages. As most selections are based on binding, factors that improve binding in positive clones, such as increased display levels (leading to increased avidity in single clones and a statistical population advantage) and increased affinity, will be favoured, these being therefore desired properties positively selected for. However, negative factors that work towards the elimination of some positive clones, such as antibody toxicity and deleterious effects on bacterial growth rates, are often more important than the positive effects of good binding. In addition, irrelevent clones, either present within the primary library, or arising during repeated growth cycles, having non-specific growth advantages over clones displaying functional antibodies, or clones recognising irrelevent or contaminating epitopes, can dominate a selection after a few selection rounds without any binding activity for the desired antigen (de Bruin et al., 1999).

This conflict between positive and negative selective forces, and the growth of irrelevent clones, is the reason that the full theoretical diversity present within a library is often practically inacessible. This represents an interesting point in the molecular diversity technologies: to what extent is the diversity present in the initial library being sampled and identified using any particular selection method?

The importance of negative selective forces is illustrated in the first series of experiments, in which it is clearly shown that continuing selection cycles reduce diversity, even if the percentage of positive clones may increase. This is in line with expectation, and experiments (data not shown) in our laboratory have shown that diversity can eventually reduce to only one or two clones, which may or may not bind antigen, if selection is continued indefinitely.

The effect of changes in selection strategy were examined in the next series of experiments, in which antibodies were selected against a panel of 11 different antigens from a large phage antibody library

using two different selection methodologies. The identities of the antibodies isolated were compared by PCR fingerprinting, a method shown to closely mirror true diversity as determined by DNA sequencing (Sblattero and Bradbury, 2000). The first selection method is a modification of the standard method in which antigen is coupled to a plastic immunotube, whereas the second method involves the use of a microtitre format. The differences between the two methods are detailed in Table 1. In general, the microtitre method uses lower amounts of antigen, a more dilute phage library and more stringent washing. Surprisingly, these relatively minor differences resulted in very different selection outcomes for the 11 different antigens. Perhaps the most important point is that most of the antibodies selected tended to be specific to the method used, and were not found in the other method. Taking the group of 130 antibodies as a whole, less than 10% were found with both methods, although this varies from a maximum of 23% for antibodies selected against the immunoglobulin domains of RPTP Sigma to a minimum of 0% for five of the antigens. This is in contrast to the 13–75% (with a mean of 30%) of common antibodies found when selection was repeated on two antigens using the two methods (Table 3b).

These results are somewhat surprising, indicating that any single selection method is likely to yield only a subset of those clones that are selectable, which in turn is likely to be an even smaller subset of those that bind the antigen of interest, but which cannot be selected.

It is difficult to say which of the two methods described is 'better'. The immunotube method tended to isolate more antibodies per antigen (mean 7.5) than the microtitre method (mean 4.9). However, this was very variable, with some antigens (e.g. 3, 4, 7 and 11—Yotiao, SNK, STAM and RPTP sigma) selecting more antibodies when the microtitre method was used, and others (e.g. 2, 5, 9 and 10—PDZ domain of densin 180, Ig domains of RPTP sigma, SPAR and glutamate receptor) being much better served by the immunotube method. The microtitre method has the advantage of being extremely convenient, allowing selection against up to 96 different antigens simultaneously, with the same quantity of library as a single immunotube selection, and as such, could form a first pass selection method, or an additional one to be carried out in parallel. This is likely to prove very useful in the genome project, should high throughput methods to create selectors based on gene products be developed, with the list of antigens against which antibodies have been selected using this method in Table 4 providing suitable testimony for its effectiveness.

In summary, full exploration of sequence space is difficult due to the constraints imposed by the selection and amplification method employed. This is likely to be true for phage-based methods, as well as in vitro methods, such as ribosome display (Hanes and Plückthun, 1997; Hanes et al., 1998). However, using a diversity of selection strategies, as well as a diversity of different forms of antigen (where this is possible) exploration of sequence space is likely to be more complete, and yields a far wider range of binding antibodies.

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References

Apperson, M.L., Moon, I.S., Kennedy, M.B., 1996. Characterization of densin-180, a new brain-specific synaptic protein of the O-sialoglycoprotein family. J. Neurosci. 16, 6839–6852.

Becerril, B., Poul, M.A., Marks, J.D., 1999. Toward selection of internalizing antibodies from phage libraries. Biochem. Biophys. Res. Commun. 255, 386–393.

Bender, E., Pilkington, G.R., Burton, D.R., 1994. Human monoclonal Fab fragments from a combinatorial library prepared from an individual with a low serum titer to a virus. Hum. Antibodies Hybridomas 5, 3–8.

Bradbury, A., Persic, L., Werge, T., Cattaneo, A., 1993. From gene to antibody: the use of living columns to select specific phage antibodies. BioTech 11, 1565–1569.

Cai, X., Garen, A., 1995. Anti-melanoma antibodies from melanoma patients immunized with genetically modified autologous tumor cells: selection of specific antibodies from single-chain Fv fusion phage libraries. Proc. Natl. Acad. Sci. U. S. A. 92, 6537–6541.

de Bruin, R., Spelt, K., Mol, J., Koes, R., Quattrocchio, F., 1999. Selection of high-affinity phage antibodies from phage display libraries. Nat. Biotechnol. 17, 397–399.

de Haard, H.J., van Neer, N., Reurs, A., Hufton, S.E., Roovers, R.C., Henderikx, P., de Bruine, A.P., Arends, J.W., Hoogenboom, H.R., 1999. A large non-immunized human Fab frag-

ment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. J. Biol. Chem. 274, 18218–18230.

de Kruif, J., Terstappen, L., Boel, E., Logtenberg, T., 1995. Rapid selection of cell subpopulation-specific human monoclonal antibodies from a synthetic phage antibody library. Proc. Natl. Acad. Sci. U. S. A. 92, 3938–3942.

Duenas, M., Malmborg, A.C., Casalvilla, R., Ohlin, M., Borrebaeck, C.A., 1996. Selection of phage displayed antibodies based on kinetic constants. Mol. Immunol. 33, 279–285.

Figini, M., Obici, L., Mezzanzanica, D., Griffiths, A., Colnaghi, M.I., Winter, G., Canevari, S., 1998. Panning phage antibody libraries on cells: isolation of human Fab fragments against ovarian carcinoma using guided selection. Cancer Res. 58, 991–996.

Gao, Q., Srinivasan, S., Boyer, S.N., Wazer, D.E., Band, V., 1999. The E6 oncoproteins of high-risk papillomaviruses bind to a novel putative GAP protein, E6TP1, and target it for degradation. Mol. Cell. Biol. 19, 733–744.

Griffiths, A.D., Malmqvist, M., Marks, J.D., Bye, J.M., Embleton, M.J., McCafferty, J., Baier, M., Holliger, K.P., Gorick, B.D., Hughes-Jones, N.C., Hoogenboom, H.R., Winter, G., 1993. Human anti-self antibodies with high specificity from phage display libraries. EMBO J. 12, 725–734.

Griffiths, A.D., Williams, S.C., Hartley, O., Tomlinson, I.M., Waterhouse, P., Crosby, W.L., Kontermann, R.E., Jones, P.T., Low, N.M., Alison, T.J., Prospero, T.D., Hoogenboom, H.R., Nissim, A., Cox, J.P.L., Harrison, J.L., Zaccolo, M., Gherardi, E., Winter, G., 1994. Isolation of high affinity human antibodies directly from large synthetic repertoires. EMBO J. 13, 3245–3260.

Hanes, J., Plückthun, A., 1997. In vitro selection and evolution of functional proteins by using ribosome display. Proc. Natl. Acad. Sci. U. S. A. 94, 4937–4942.

Hanes, J., Jermutus, L., Weber-Bornhauser, S., Bosshard, H.R., Plückthun, A., 1998. Ribosome display efficiently selects and evolves high-affinity antibodies in vitro from immune libraries. Proc. Natl. Acad. Sci. U. S. A. 95, 14130–14135.

Hawkins, R.E., Russell, S.J., Winter, G., 1992. Selection of phage antibodies by binding affinity: mimicking affinity maturation. J. Mol. Biol. 226, 889–896.

Hoogenboom, H.R., Lutgerink, J.T., Pelsers, M.M., Rousch, M.J., Coote, J., Van Neer, N., De Bruine, A., Van Nieuwenhoven, F.A., Glatz, J.F., Arends, J.W., 1999. Selection-dominant and nonaccessible epitopes on cell-surface receptors revealed by cell-panning with a large phage antibody library. Eur. J. Biochem. 260, 774–784.

Jin, H., May, M., Tranebjaerg, L., Kendall, E., Fontan, G., Jackson, J., Subramony, S.H., Arena, F., Lubs, H., Smith, S., Stevenson, R., Schwartz, C., Vetrie, D., 1996. A novel X-linked gene, DDP, shows mutations in families with deafness (DFN-1), dystonia, mental deficiency and blindness. Nat. Genet. 14, 177–180.

Johns, M., George, A.J., Ritter, M.A., 2000. In vivo selection of sFv from phage display libraries. J. Immunol. Methods 239, 137–151.

Krebber, C., Spada, S., Desplancq, D., Plückthun, A., 1995. Co-selection of cognate antibody-antigen pairs by selectively infective phages. FEBS Lett. 377, 227–231.

Lin, J.W., Wyszynski, M., Madhavan, R., Sealock, R., Kim, J.U., Sheng, M., 1998. Yotiao, a novel protein of neuromuscular junction and brain that interacts with specific splice variants of NMDA receptor subunit NR1. J. Neurosci. 18, 2017–2027.

Llorente, M., Sanchez-Palomino, S., Manes, S., Lucas, P., Kremer, L., De Alboran, I.M., Toran, J.L., Alcami, J., Del Real, G., Martinez, A.C., 1999. Natural human antibodies retrieved by phage display libraries from healthy donors: polyreactivity and recognition of human immunodeficiency virus type 1gp120 epitopes. Scand. J. Immunol. 50, 270–279.

Lu, J., Sloan, S.R., 1999. An alternating selection strategy for cloning phage display antibodies. J. Immunol. Methods 228, 109–119.

Malmborg, A.-C., Duenas, M., Ohlin, M., Soderlind, E., Borrebaeck, C., 1996. Selection of binders from phage displayed antibody libraries using the BIAcore biosensor. J. Immunol. Methods 198, 51–57.

Malmborg, A.-C., Söderlind, E., Frost, L., Borrebaeck, C.A.K., 1997. Selective phage infection mediated by epitope expression on F pilus. J. Mol. Biol. 273, 544–551.

Marks, J.D., Hoogenboom, H.R., Bonnert, T.P., McCafferty, J., Griffiths, A.D., Winter, G., 1991. By-passing immunization. Human antibodies from V-gene libraries displayed on phage. J. Mol. Biol. 222, 581–597.

Mutuberria, R., Hoogenboom, H.R., van der Linden, E., de Bruine, A.P., Roovers, R.C., 1999. Model systems to study the parameters determining the success of phage antibody selections on complex antigens. J. Immunol. Methods 231, 65–81.

Nusser, Z., Lujan, R., Laube, G., Roberts, J.D., Molnar, E., Somogyi, P., 1998. Cell type and pathway dependence of synaptic AMPA receptor number and variability in the hippocampus. Neuron 21, 545–559.

Palmer, D.B., George, A.J., Ritter, M.A., 1997. Selection of antibodies to cell surface determinants on mouse thymic epithelial cells using a phage display library. Immunology 91, 473–478.

Pasqualini, R., Ruoslahti, E., 1996. Organ targeting in vivo using phage display peptide libraries. Nature 380, 364–366.

Perelson, A.S., Oster, G.F., 1979. Theoretical studies of clonal selection: minimial antibody repertoire size and reliability of self non-self discrimination. J. Theor. Biol. 81, 645–670.

Persic, L., Horn, I.R., Rybak, S., Cattaneo, A., Hoogenboom, H.R., Bradbury, A., 1999. Single-chain variable fragments selected on the 57–76 p21Ras neutralising epitope from phage antibody libraries recognise the native protein. FEBS Lett. 443, 112–116.

Pulido, R., Serra-Pages, C., Tang, M., Streuli, M., 1995. The LAR/PTP delta/PTP sigma subfamily of transmembrane protein-tyrosine-phosphatases: multiple human LAR, PTP delta, and PTP sigma isoforms are expressed in a tissue-specific manner and associate with the LAR-interacting protein LIP.1. Proc. Natl. Acad. Sci. U. S. A. 92, 11686–11690.

Sauer, B., Henderson, N., 1988. The cyclization of linear DNA in *Escherichia coli* by site-specific recombination. Gene 70, 331–341.

Sblattero, D., Bradbury, A., 2000. Exploiting recombination in single bacteria to make large phage antibody libraries. Nat. Biotech. 18, 75–80.

Sheets, M.D., Amersdorfer, P., Finnern, R., Sargent, P., Lindqvist, E., Schier, R., Hemmingsen, G., Wong, C., Gerhart, J.C., Marks, J.D., 1998. Efficient construction of a large nonimmune phage antibody library; the production of panels of high affinity human single-chain antibodies to protein antigens. Proc. Natl. Acad. Sci. U. S. A. 95, 6157–6162.

Siegel, D.L., Chang, T.Y., Russell, S.L., Bunya, V.Y., 1997. Isolation of cell surface-specific human monoclonal antibodies using phage display and magnetically activated cell sorting: applications in immunohematology. J. Immunol. Methods 206, 73–85.

Simmons, D.L., Neel, B.G., Stevens, R., Evett, G., Erikson, R.L., 1992. Identification of an early-growth-response gene encoding a novel putative protein kinase. Mol. Cell. Biol. 12, 4164–4169.

Takeshita, T., Arita, T., Asao, H., Tanaka, N., Higuchi, M., Kuroda, H., Kaneko, K., Munakata, H., Endo, Y., Fujita, T., Sugamura, K., 1996. Cloning of a novel signal-transducing adaptor molecule containing an SH3 domain and ITAM. Biochem. Biophys. Res. Commun. 225, 1035–1039.

Van Ewijk, W., de Kruif, J., Germeraad, W.T.V., Berendes, P., Ropke, C., Platenburg, P.P., Logtenberg, T., 1997. Subtractive isolation of phage-displayed single-chain antibodies to thymic stromal cells by using intact thymic fragments. Proc. Natl. Acad. Sci. U. S. A. 94, 3903–3908.

Vaughan, T.J., Williams, A.J., Pritchard, K., Osbourn, J.K., Pope, A.R., Earnshaw, J.C., McCafferty, J., Hodits, R.A., Wilton, J., Johnson, K.S., 1996. Human antibodies with sub-nanomolar affinities isolated from a large non-immunised phage display library. Nat. Biotechnol. 14, 309–314.