



SELECTION OF PHAGE DISPLAYED ANTIBODIES BASED ON KINETIC CONSTANTS

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Abstract—The display of antibody fragments on the surface of filamentous bacteriophages and the selection of binders from antibody libraries have provided powerful tools to generate human antibodies. We reported recently a new concept (SAP system) for the selection of specific phages by linking antigenic recognition and phage replication, using a soluble fusion protein containing the antigen and a fragment of the M13 coat protein 3. In this investigation, a model library has been composed using six different antibody fragments which were characterized individually regarding their k_{ass} , k_{diss} and K_a . All Fab fragments were specific for a 15 amino acid region of the V3 loop of gp120 (HIV-1). We demonstrated that the SAP system could discriminate between the kinetic parameters of each clone, using different selection strategies. Phages expressing high affinity clones were selected preferentially using low doses of antigen but clones of lower affinity also could be selected by increasing the antigen concentration or using a preselection procedure. Phages expressing antibody fragment with high association or low dissociation rate constants were retrieved by utilizing short contact times between antigen and antibody or antigen-chase conditions. Copyright © 1996 Elsevier Science Ltd.

Key words: Antibody, phage display, antigen-specific selection, SAP, dissociation, association, rate constant.

INTRODUCTION

The display of molecular fragments on the surface of a filamentous bacteriophage (Smith, 1985) by fusion to a phage coat protein has provided a powerful tool to select antibodies of predefined binding specificity from V-gene repertoires (Parmley and Smith, 1988; McCafferty *et al.*, 1990; Barbas *et al.*, 1991; Chang *et al.*, 1991; Hoogenboom *et al.*, 1991; Söderlind *et al.*, 1993; Winter *et al.*, 1994). Starting from antibody repertoires derived from immunized mice or humans, it has been possible also to select antibody fragments with high affinities (Clackson *et al.*, 1991; Barbas *et al.*, 1992a, 1992b). Since the source of immunized human B cells is a limiting factor, alternative approaches with naive (Marks *et al.*, 1991) or synthetically assembled variable regions (Hoogenboom and Winter, 1992; Söderlind *et al.*, 1995) have been explored. The majority of antibodies derived from these sources exhibited affinities in the μM range, although the possibility to find high affinity antibodies from large primary repertoires has been postulated (Perelson and Oster, 1979), even though the required size of such libraries is not known presently. However, Griffiths *et al.*

(1994) reported recently the construction of a synthetic library of 10^{10} members, using the Cre/loxP system of recombination (Waterhouse *et al.*, 1993), and these investigators could then select antibodies with nM affinity.

For large libraries, the selection procedure plays a crucial role in the process of retrieving the few copies of a desired antibody fragment from a large pool of specificities. We described recently a powerful technology, called SAP (Selection and Amplification of Phages) (Dueñas and Borrebaeck, 1994). From libraries constructed using primary and secondary *in vitro* immunized human B cells, containing fragments with affinity constants (K_a) in the range of 10^8 – $10^{10}/\text{M}$, we now demonstrate that Fab fragments can be selected based on their individual kinetic rate constants. This was performed by establishing a model phage library of six clones with distinct kinetic parameters. By varying time of antibody–antigen interaction or by using antigen-chase conditions, rapid selections based on association and dissociation rate could be obtained. These procedures thus will allow selection of antibody fragments from large libraries with properties directly tailor-made for particular applications.

MATERIALS AND METHODS

Strains, phages and reagents

Escherichia coli strains Y 1089 and XL1-Blue were used for the expression of the fusion protein and phagemid

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preparation, respectively. The M13MDA3.2, prepared according to Dueñas and Borrebaeck (1995), was used as helper phage. Polymerase chain reaction (PCR) was performed with reagents and enzymes from Perkin-Elmer/Roche (Braunschweig, NJ, U.S.A.) using thermocyclers from Cetus Corporation (Emeryville, CA, U.S.A.). Oligonucleotides were synthesized using a PCR-mate from Applied Biosystems (Warrington, U.K.). The phage displayed Fab fragments used were described recently by Dueñas *et al.* (1996). In order to facilitate the discussion, "Hi" or "Lo" was added to each clone number, referring to the K_a of each Fab fragment.

Titration of individual phage stocks

The six phagemids encoding specific antibody fragments were electroporated (Porter, 1993) into XL1-Blue cells. Phage stocks were prepared as described recently (Dueñas *et al.*, 1996). Briefly, single colonies were grown overnight in $2 \times$ YT medium, containing ampicillin and tetracycline (100 and 50 $\mu\text{g}/\text{ml}$, respectively). Following a 1:100 dilution in fresh medium, the cultures were grown to $\text{OD}_{550} = 0.5$ and the helper phage (M13MDA3.2) was added in a ratio of 1:10 (cell:phage). After 1 hr, kanamycin (34 $\mu\text{g}/\text{ml}$) was added. After an additional 16 hr at 37°C , cultures were centrifuged at $8000g$ and the phage-containing supernatant was filtered through a 0.2 μm filter (Microgen Inc., Laguna Hills, CA, U.S.A.). Different dilutions of the phages were incubated with different concentration of the fusion protein overnight at room temperature. The antigenic epitope was a 15 amino acid peptide derived from the V3 loop of gp 120 of the LAI strain (Putney *et al.*, 1986). Cells were infected and plated on YT-agar plates containing ampicillin. Colony forming units (cfu) were determined after 16 hr.

Dose-dependent selection of specific antibody fragments

The mixture containing phage displayed Fab fragments was incubated with three different concentrations of the fusion protein (1, 1.5 and 2%) at room temperature. At different times of incubation (1–16 hr), cells were infected and plated on YT-agar, containing 100 $\mu\text{g}/\text{ml}$ ampicillin. After overnight incubation at 37°C , the cfu were determined. A phage stock displaying an antibody fragment specific for hen egg lysozyme was used as negative control.

Selection on association/dissociation rate constants

A mixture of six antibody-expressing phage clones (60 cfu each) was incubated with 0.5% soluble protein 3-antigenic epitope fusion protein, at room temperature. At various time points (from 0.5 to 16 hr), bacteria were infected for 15 min at room temperature and plated on YT-agar containing ampicillin (100 $\mu\text{g}/\text{ml}$) and grown overnight at 37°C . For the antigen-chase experiment, a soluble competing peptide comprising only the antigenic epitope (15 amino acids) was added at 0 or 60 μM final concentration to the phage–fusion protein mixture after 16 hr of incubation. The resulting clones were evaluated

by PCR on single colonies using primers specific for each particular clone.

Depletion of high affinity fragments from the mixture

A first round of selection–infection using an overnight incubation was prepared as described and the remaining phage-containing supernatant was incubated again with a fresh 0.5% (v/v) fusion protein-containing supernatant. At different times (0.5, 1 and 2 hr), 100 μl of XL1-Blue cells in exponential phase were infected and plated on YT-agar ampicillin plates. After 16 hr, colonies were analysed by PCR.

Oligonucleotides designed for specific amplification

An oligonucleotide specific for the C_k domain was used as 3'-primer: CCAAGCTTCATCAGATGGCGGGAGAT. To allow differentiation of each clone, the following oligonucleotides were used as 5'-primer:

- Clone 1: CCATCAGTAGTCTGCAGTCC;
- Clone 8: GTCCTCGCAACTGCAGCGGCGGCA-CCAGCGATGGCCTCCTATGTGCTG-ACTCAGCCACC;
- Clone 23: GCCTCCTCCAGGGCCCCCTGA;
- Clone 24: GAACACACTGGTCACCCACG;
- Clone 25: TACTTGTCGGGCGAGACAGA;
- Clone 30: ACTGGGGGAGTACCAAAAAC.

Single colony PCR

The screening of single colonies was performed by a PCR protocol. Forty independent colonies were evaluated for each incubation condition. We prepared a mixture of the seven different primers described above. One of these annealed to the C_k domain and the others annealed specifically to one of the different clones. The PCR reaction tube contained 25 μl of a solution of 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 2 mM MgCl_2 ; 1 μM of each primer; 200 μM of dNTP and 50 U/ml of AmpliTaq polymerase (Perkin-Elmer, Norwalk, CT, U.S.A.). A single colony was picked to each tube, mixed and covered with 50 μl of mineral oil. The PCR was set up in 30 cycles of 94°C for 1 min, 60°C for 30 sec and 72°C for 1 min. After the PCR, the samples were evaluated by 2% agarose–TAE electrophoresis and the size of the band was visualized by ethidium bromide staining. Since the 5'-primer specific for each clone was designed to anneal to different regions of the genes, the amplification resulted in fragments of different sizes, allowing an identification of each clone.

Kinetic determination

The BIAcoreTM biosensor system (Pharmacia Biosensor AB, Uppsala, Sweden) was used to determine the kinetic parameters for the interactions between the Fab fragments and pB1 as discussed previously (Dueñas *et al.*, 1996).

Statistical evaluation

The result of the selection experiments in relation to kinetic parameters were analysed with the CSS: Statistica software package (Release 3.0A; Stat Soft, Tulsa, OK, U.S.A.). The mean of each frequency distribution for each kinetic constant and the time of antigen-antibody incubation was established and the Spearman coefficient (R) (Kempi, 1985) for each distribution was calculated. In order to determine the level of significance of the correlation, a Student's t -test was applied. Significant differences were evaluated at the 99% confidence level.

RESULTS

Establishing a model phage library

Six different Fab fragments, characterized previously regarding their individual binding characteristics (Dueñas *et al.*, 1996) (Table 1), were displayed on phages. Each displayed Fab fragment was prepared as a phage stock and all phage stocks were titrated individually using two different concentrations of fusion protein (antigen-p3) in the SAP procedure (Fig. 1). Thus, a low antigen

dose preferentially selected antibody fragments exhibiting high affinity constant (clones 24Hi, 25Hi and 30Hi), while higher antigen doses also recruited Fab fragments exhibiting lower affinity constants (clones 1Lo, 8Lo and 23Lo). The results suggested a dose-dependent selection of Fab fragment, although the efficiency of infection can be envisaged to be the result, not only of the K_a , but also the frequency of phages and the fraction of phages actually displaying the specific antibody fragment. A model phage library was subsequently established, containing approximately the same amount of infectious units of each clone. The dilution of the phage stocks was adjusted so that approximately 60 cfu/100 μ l of each clone was present when 0.5% fusion protein was used.

Section on association/dissociation rate constants from the model library

Using the model library described above, we investigated the time dependence of antigen-antibody binding and selection. During a first interval of 16 hr (Fig. 2), the frequency of each clone was determined after different times of interaction between displayed Fab and fusion protein. Firstly, only clones with the highest affinity constants (24Hi, 25Hi and 30Hi) were selected. This discrimination between clones having high and low affinity constants was statistically significant ($p < 0.001$) and confirmed that K_a was the only parameter that correlated with the frequency of the selected clones. Secondly, among the clones with high affinity constants, clone 30Hi with the highest association rate constant (k_{ass}) was selected preferentially during the first 30 min of incubation. In this time period, k_{ass} was the major selective force as shown by a statistical evaluation ($p < 0.001$). After reaching equilibrium, there was no statistical

Table 1. Clones used for the kinetic-based SAP selection

Clone number	Association rate constant (/M/s)	Dissociation rate constant (/s)	Affinity constant (/M)
1Lo	1.7×10^5	3.9×10^{-4}	4.3×10^8
8Lo	6.0×10^4	1.2×10^{-4}	5.0×10^8
23Lo	6.2×10^3	2.3×10^{-5}	2.7×10^8
24Hi	1.2×10^4	7.8×10^{-6}	1.5×10^9
25Hi	6.3×10^4	1.6×10^{-5}	3.9×10^9
30Hi	2.1×10^5	8.4×10^{-5}	2.5×10^9

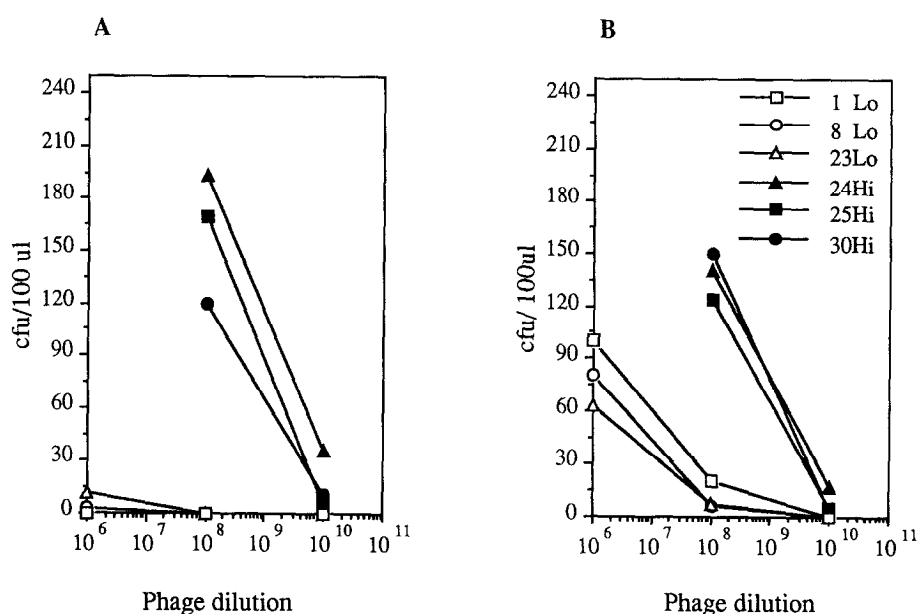


Fig. 1. Effect of different fusion protein concentrations on the selected phage-displayed Fab fragments, using 0.1% (v/v) (A) and 1% (B) of fusion protein-containing supernatant. Open symbols indicate low affinity antibodies and closed symbols indicate high affinity antibodies.

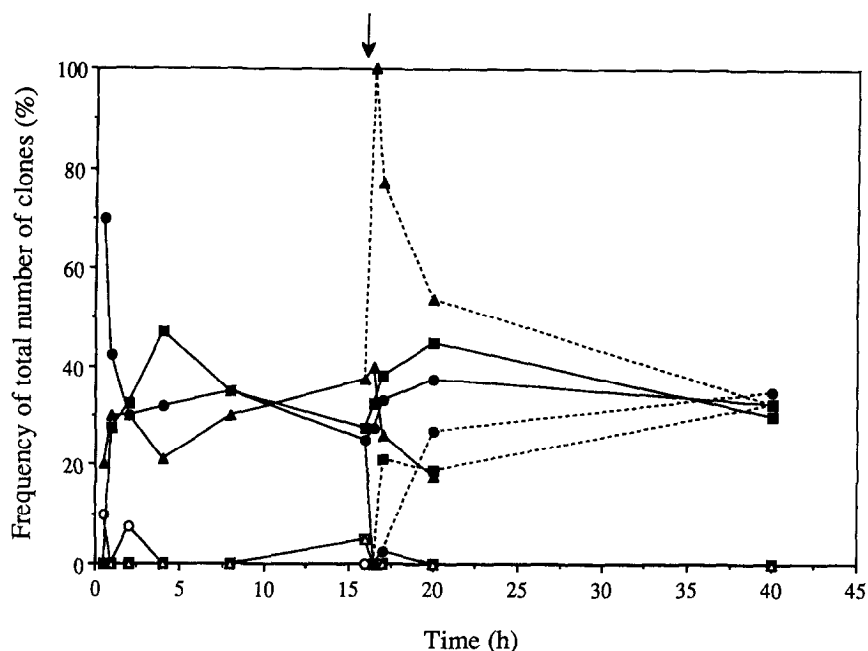


Fig. 2. Kinetic, antigen-specific selection from the model library, using 0.5% (v/v) of fusion protein-containing supernatant. After 16 hr of incubation (see arrow) a competing antigen was added (dashed line) to the high affinity clones. This resulted in selection of the clone with the lowest k_{diss} value. Control experiments with no addition of competing antigen at 16 hr are shown also (solid lines).

difference between the frequency of selected clones and their k_{diss} .

Addition of a soluble, competing antigen (Fig. 2) after 16 hr changed the selection preference to the fragment with the lowest dissociation rate constant (k_{diss}) (clone 24Hi) among the high affinity clones. An equilibrium was reached after an additional 4 hr of antigenic chase. The statistical analysis of this experiment showed a correlation ($p < 0.01$) between k_{diss} and the frequency of selected clones during the first 2 hr of antigenic chase.

Strategies to select low affinity clones in the presence of high affinity clones

The effect of fusion protein concentrations and time of interaction on the SAP procedure was then evaluated. It was evident that increasing concentrations of the fusion protein compromised the specificity of the amplification (Fig. 3) i.e. when the dose was increased to 2%, a non-specific background could be observed. The number of selected clones also decreased as a function of incubation time between phage and fusion protein, indicating a decreasing stability of this complex with time. Consequently, only the effect of the lowest concentrations of fusion protein were evaluated, since the background of non-specific infection was low under those conditions. The model library was used for selection with 1% of fusion protein. It was possible to detect the low affinity clones although high affinity clones were still selected preferentially [Fig. 4(A)]. However, when 1.5% of fusion protein was used, both low and high affinity clones were selected with a 2 hr incubation time [Fig. 4(B)].

In a further attempt to investigate approaches to retrieve low affinity clones in the presence of high affinity clones, a 1:10 mixture of high to low affinity clones was

prepared. The selection of a particular phage was influenced by its frequency in the initial mixture [Fig. 5(A)]. During the first 30 min, as was observed also for the high affinity clones [Fig. 2(A)], the selection was driven by k_{ass} , since mainly clone 1Lo displaying a high on-rate was reached.

Thus, low affinity clones could be retrieved if present in excess and we now investigated selection from the

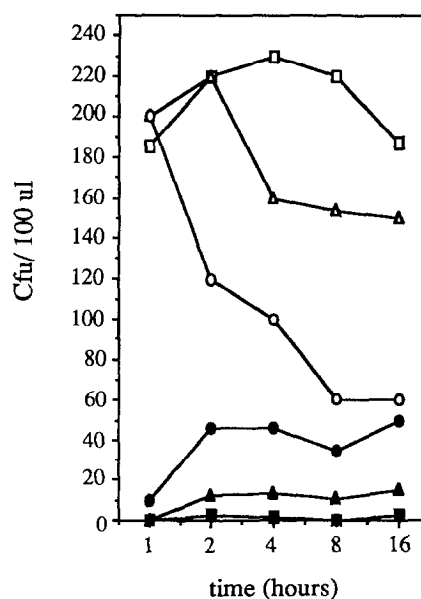


Fig. 3. Effect of the fusion protein concentration [1% ($\square$), 1.5% ($\triangle$) and 2% ($\circ$)] on the number of selected clones. The open symbols represent selection from the mixture of specific phage-displayed antibodies and the closed symbols represent the non-specific infection obtained by using phage-displayed antibody fragments specific for hen egg lysozyme.

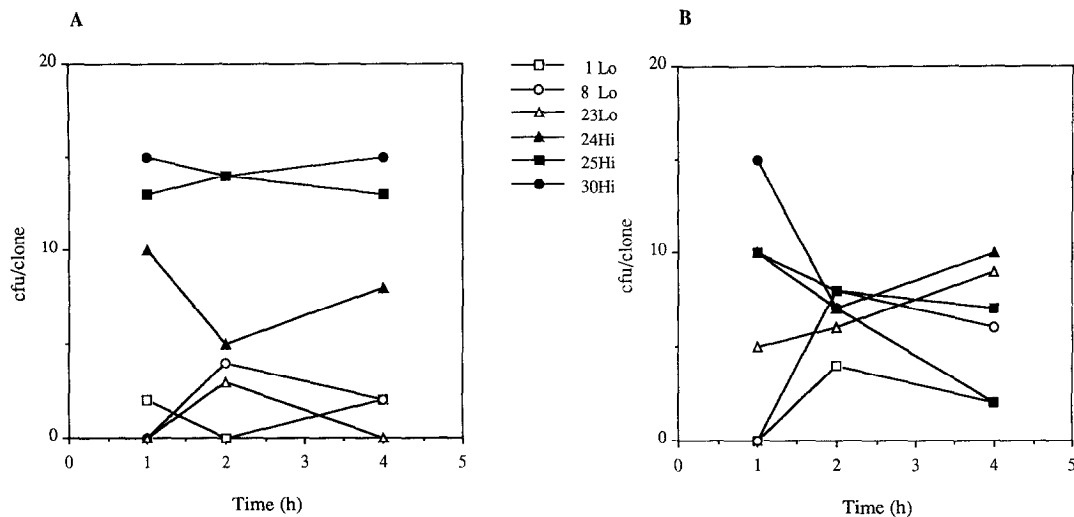


Fig. 4. Effect of fusion protein-dosage on the selection of low/high affinity clones from the model library. Panel A represents the frequency of each clone selected by using 1% (v/v) and panel B represents the selection after incubation with 1.5% (v/v) of the fusion protein, respectively.

model library, where each clone was equally represented. High affinity clones were removed first by preselection, using one round of selection (0.5% fusion protein). Phages still remaining in the supernatant were subsequently selected following addition of another 0.5% (v/v) of fusion protein. It was evident that after the pre-selective removal of the high affinity clones, low affinity clones could be selected easily in the second round [Fig. 5(B)].

DISCUSSION

In the immune system, the selection and proliferation process appears to be capable of selecting specific B cells from potential repertoires of 10^{11-12} . It is able also to enrich for B cells displaying antibodies with considerably improved binding affinities after a process of somatic

mutations and selection (Kocks and Rajewsky, 1988). In libraries, antibodies displayed on phages have been selected by binding to antigen-coated plates (Barbas *et al.*, 1991; Marks *et al.*, 1991), chromatography matrices (McCafferty *et al.*, 1990), cells (Marks *et al.*, 1993) or to biotinylated antigen followed by streptavidin capture (Hawkins *et al.*, 1992). In this way, enrichment factors of 20–1000 per round of selection have been reported. To take advantage of the link between recognition and replication used by the immune system, we have developed a novel selection system (Dueñas and Borrebaeck, 1994), which links antibody–antigen recognition and phage replication. This system is based on a soluble fusion protein comprising the antigenic epitope and the fragment of protein 3 of M13 which is responsible for the recognition of F' pili. The antibody–antigen interaction provide, to a non-infectious antibody-displaying phagemid particle,

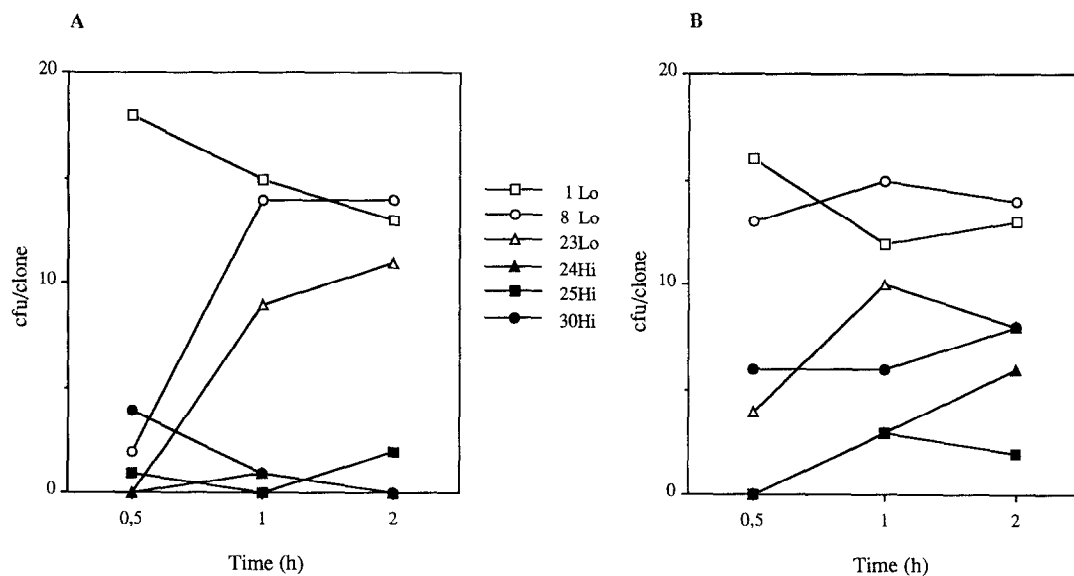


Fig. 5. Retrieval of low affinity clones by SAP selection. Panel A represents the selection from a mixture containing 10 times excess of low affinity clones and panel B the selection of low affinity clones following selective removal of high affinity clones by a previous round of selection.

the necessary element for infection and subsequent phage replication. Enrichment factors of $> 10^5$ per round of selection were obtained (Dueñas and Borrebaeck, 1994).

Using conventional selection systems, one can under some circumstances select phages based on K_a and k_{diss} of the displayed antibody fragment, by manipulating the antigen concentration during selection (Barbas *et al.*, 1992a; McCafferty *et al.*, 1992). However, in these systems, high affinity antibodies could be difficult to elute and clones with high k_{diss} might be lost.

Here we show for the first time, by the homogeneous SAP approach, how antibody fragments could be selected in a dose-dependent manner and how fragments exhibiting different kinetic parameters were selected. Thus, we demonstrate that fragments displaying high K_a are selected preferentially independent of the time of antibody-antigen interaction. Furthermore, within the high affinity group, during the first time period we could selectively enrich for fragments with fast association rate. After the equilibrium was established, an addition of competing free antigen (not fused to the protein 3 fragment) favoured the selection of those antibodies displaying a slow dissociation rate. These data support a kinetic manipulation of the selection process by varying the time of antigen binding to the displayed antibody fragments and by antigen-chase conditions.

To select low affinity antibodies in the presence of high affinity clones the initial strategy was to increase the concentration of the fusion protein. This resulted in a negative effect of the infectivity by allowing non-specific infection, consequently compromising the specificity of the selection process. Another effect, using this strategy, was the reduction in specific infection observed after the first hours of incubation. However, using an intermediate level of fusion protein and short incubation times, we were able to select both high and low affinity antibodies present in the model library. On the other hand, a more efficient way to retrieve low affinity clones from the mixture was first to deplete the high affinity clones by a preselection step and then, in a subsequent round of selection, it was possible to retrieve efficiently the low affinity clones.

In summary, we have demonstrated that phage displayed antibodies can be selected directly based on their individual association/dissociation rate constants as well as on their affinity constants, using a homogeneous selection approach.

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