THE SAP AS A HOMOGENEOUS AND KINETIC BASED SELECTION SYSTEM FOR ACTIVE PROTEINS IN COMBINATORIAL LIBRARIES

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The development of antibody libraries in *Escherichia coli*, the use of filamentous bacteriophages and strategies to mimic the humoral response has technologically opened, a new stage in immunological studies. In particular the cuestion of antibody libraries has been important and today large germline repertoires have been selected and analized. The major limitation of this procedure, seems to be the low affinity of the selected antibodies and the low copy number of each binder in the library. To overcome that problem, *in vitro* affinity maturation and different selection strategies have been used.

The most common strategy for selection of antibodies from phage displayed libraries is panning (1, 2) where the antigen of interest is coated onto a solid surface and bound phage are, after extensive washing, eluted. A similar principle holds true for the selection using antigen coated magnetic beads or immunotubes (3). The antigen can also be coupled to an affinity chromatography column and thus serve as means for selection (4, 5). Letting the selection take place in solution, in a homogeneous system, and linking the selection to infection would even further mimick phage display to the *in vivo* situation of how B cells with antibodies on their surfaces are clonally expanded upon antigen stimulation.

We developed a novel system of antibody selection based on the same principle as clonal selection in the immune system, the SAP (selection and amplification of phages) system. Figure 1 shows a schematic representation of how the system enriched the specific phagemide particles over the non-specific phages. A designed mutant helper phage mediated production of non-infectious phagemid particles, which when combined with a fusion protein comprising the antigen and the N-terminal fragment of protein 3, responsible for pili recognition, permitted clonal expansion of a specific clone. This means that only specific phages are infective. This system provides enrichment factors of up to 10^5 in a single round of selection of a model library (Table 1) and has proven to be, at least, two order of magnitude more efficient than the conventional panning technique when compared in the selection of a naive library (6).

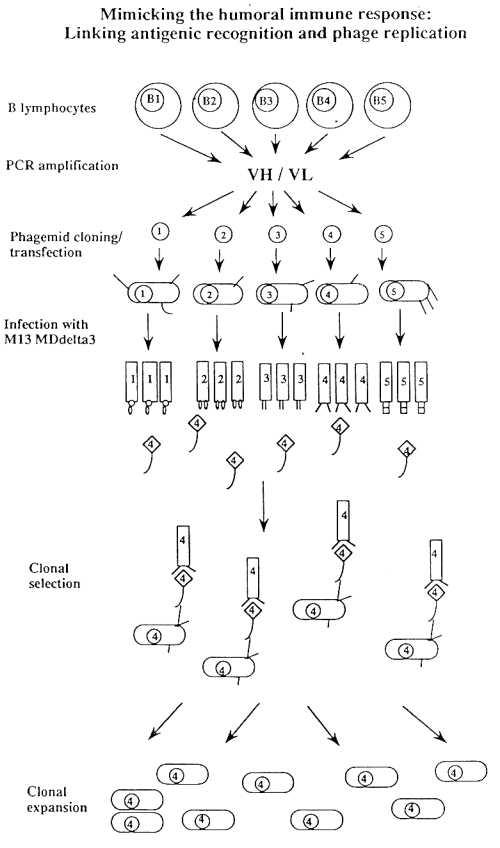

Figure 1. The SAP system in combinatorial libraries. Only the specific phages replicates after each round of selection.

Table 1. Specific enrichment using SAP. Enrichment factor = initial ratio/final ratio. Clonal mixture: PEXmide HEL/PEXmide phox/ PEXmide LPS.

	Initial ratio	Final ratio	Enrichment
First	$1/3 \times 10^4$	82/20	1.2×10^5
round	$1/3 \times 10^5$	49/59	2.5×10^5
of	$1/3 \times 10^6$	4/104	1.1×10^5
selection	$1/3 \times 10^7$	0/108	n.d.
Second	$1/3 \times 10^8$	103/5	6.1×10^9
round	$1/3 \times 10^9$	55/53	3.1×10^9
of	$1/3 \times 10^{10}$	16/92	5.2×10^9
selection	$1/3 \times 10^{11}$	2/106	5.6×10^9
	$1/3 \times 10^{12}$	0/108	n.d.

1. Barbas III CF, Kang AS, Lerner RA, Benkovic SJ. Assembly of combinatorial antibody libraries on phage surface: the gene III site. Proc Natl Acad Sci USA 1991;88:7978-7982.

2. Marks JD, Griffiths AD, Malmqvist M, Clackson T, Bye JM, Winter G. By-passing immunization: building high affinity human antibodies by chain shuffling. Bio/Technology 1992;10:779-783.

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4. McCafferty J, Griffiths AD, Winter G, Chiswell DJ. Phage antibodies: filamentous phage displaying antibody variable domains. Nature 1990;348: 552-554.

5. Clackson T, Hoogenboom HR, Griffiths AD, Winter G. Making antibody fragments using phage displayed libraries. Nature 1991;352:624-628.

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To demonstrate the potential of phage library and the SAP selection, we used the technology to evaluate a human *in vitro* immune response. We constructed antibody libraries from naive, primary and secondary *in vitro* immunized B cells and characterized the antibody repertoire from these responses from two seronegative donors. In an *in vivo* immune response it has been shown that during the first antigenic stimulation a specific, low affinity response develops, generating mainly IgM antibodies, with sequences very similar to the germline from which they originated. The secondary response is characterized by high affinity antibodies, which has class switched to IgG, containing a characteristic pattern of hypermutations concentrated to the CDR regions. The antibody fragments from these libraries were analized and their mutational pattern was similar to what has been reported for *in vivo* responses and the replacement/silent mutation ratios suggested that somatic hypermutations had taken place (results not shown). Subsequently, the protein analysis showed an affinity maturation of the response occurring as a consequence of the immunization steps (Figure 2). This was determined by an increase in the affinity constant, mainly due to a decrease in the dissociation rate constant.

Elution of bound phage in any of the selection methods comprising a solid phase can be varied so as to vary the affinity of the selected phage displayed antibody. Mild elution conditions will elute phages that have bound weakly and more stringent elution will also elute phages that bind with higher affinity. Garrard, *et al.* (7) speculate that the washing procedure selects for phage with the faster on-rates, while Marks, *et al.* (2) argue that the higher affinities selected for are due to a slower off-rate. Hawkins, *et al.* (8) managed to discriminate between antibodies with closely related affinities by incubating the phage library with small amounts of soluble biotinilated antigen such that the antigen was in excess over phage but with the concentration of antigen lower than the K_D of the antibody. Phage bound to antigen were selected using streptavidin coated magnetic beads. To select for slow off-rates they competed the interaction between phage and biotinilated antigen with non-biotinilated antigen. The biotinilated antigen that was left on phage allowed for selection with streptavidin coated magnetic beads and the k_{diss} for the selected antibodies were proven to be low.

Another approach towards modification of selection conditions to discriminate between different affinities and kinetic parameters has been performed with SAP. The use SAP has proven to select for high affinity antibody fragments (9, 10), so we decided to try to modify the conditions to select on the basis of k_{ass} and k_{diss}. A model library was created of six Fab fragments displayed on phages, with known k_{ass}, k_{diss} and K_A (Table 2). We found again, that normal SAP procedure favoured those clones of high

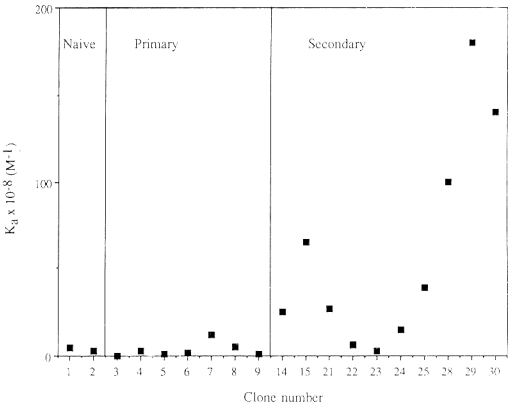

Figure 2. The affinity constants of all the antigen specific Fab clones as a function of immunization status of the PBL used for library construction. First panel: unimmunized PBL; second panel: primary *in vitro* immunized PBL; third panel: secondary *in vitro* immunized PBL.

affinity (Figure 3). If clones were selected after a short time of interaction with fusion protein, those clones expressing fast on-rates were favoured, and if a competing antigen was added after the initial interaction between phage and fusion protein, those of low off-rates were favoured. Consequently, in addition to selection based on off rates as previously described (8), we were able to select binders based on on-rates as well.

7. Garrard LJ, Yang M, O Connell MP, Kelley RF, Henner DJ. Fab assembly and enrichment in a monovalent phage display system. Bio/Technology 1991;9: 1373-1377.

8. Hawkins RE, Russell SJ, Winter G. Selection of phage antibodies by binding affinity: mimicking affinity maturation. J Mol Biol 1992;226:889-896.

Table 2. Kinetic constants of clones used for the kinetic-based SAP selection.

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

In summary, the described method for selecting active proteins from a combinatorial library provides some advantages over the previous selection methods reported. First, provides an easy and faster selection method as compared to the heterogeneous selection systems. Second, Avoids the need of purified antigen for the specific selection. Third, high efficiency in selection is provided by linking antigenic recognition and phage infectivity. And fourth, provides a unique method of selection of antibody fragments based in all their kinetic parameters.

9. Dueñas M, Chin LT, Malmborg AC, Casalvilla R, Ohlin M, Borrebaeck CAK. *In vitro* immunization of naive human B cells yields high affinity IgG antibodies as illustrated by phage display. Immunology 1996; In press.

10. Malmborg AC, Dueñas M, Ohlin M, Soderlind E, Borrebaeck CAK. Selection of binders from phage displayed antibody libraries based on dissociation rate constants using the BIAcore™ biosensor. J Immunol Methods 1996;In press.

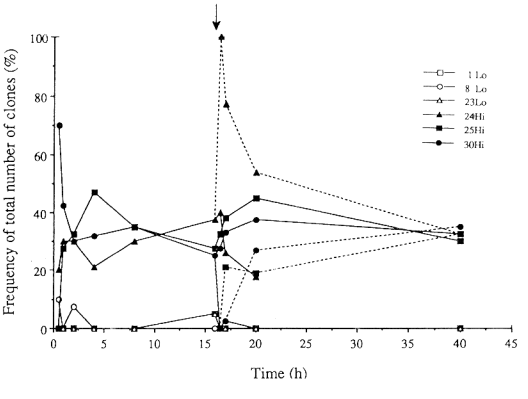

Figure 3. Kinetic, antigen-specific selection from the model library, using 0.5 % (v/v) of fusion protein-containing supernatant. After 16 h of incubation (see arrow) a competing antigen was added (dashed line). This resulted in selection of the clone with lowest k_{diss} value. Control experiments with no addition of competing antigen at 16 h are shown also (solid line). Clonal mixture: PEXmide HEL/PEXmide phox/ PEXmide LPS.