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Molecules and Cells

Antibodies against Non-Immunizing Antigens Derived from a Large Immune scFv Library

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Target-specific antibodies can be rapidly enriched and identified from an antibody library using phage display. Large, naïve antibody libraries derived from synthetic or unimmunized sources can yield antibodies against virtually any antigen, whereas libraries from immunized sources tend to be smaller and are used exclusively against the antigen of immunization. In this study, 25 scFv libraries made from the spleens of immunized rabbits, each with a size ranging from 10^8 to higher than 10^9, were combined into a single large library with > 10^{10} individual clones. Panning of this combined library yielded target-specific rabbit scFv clones against many non-immunizing antigens, including proteins, peptides, and a small molecule. Notably, specific scFv clones against a rabbit self-antigen (rabbit serum albumin) and a phosphorylated protein (epidermal growth factor receptor pTyr1173) could be isolated from the library. These results suggest that the immune library contained a significant number of unimmunized clones and that a sufficiently large immune library can be utilized similarly to a naïve library, i.e., against various non-immunizing antigens to yield specific antibodies.

INTRODUCTION

Antibody phage display is a powerful tool for rapidly and efficiently isolating target-specific antibody clones from an antibody library. Genetic diversity for the construction of antibody libraries can be obtained from synthetic degenerate oligonucleotides (Barbas et al., 1992; Griffiths et al., 1994; Rothe et al., 2008) or from B cells of immunized (Barbas et al., 1991; Clackson et al., 1991; Huse et al., 1989; McCafferty et al., 1990) or non-immunized animals (Glanville et al., 2009; Marks et al., 1991; Vaughan et al., 1996). While synthetic or non-immunized natural libraries can be used against virtually any antigen to yield antigen-specific clones, such libraries must be sufficiently large (typically having 10^9 or greater number of unique clones) and thus are difficult to construct. Compared with these naïve libraries, antibody libraries derived from immunized sources require lower diversity (10^7-10^8) (Rader et al., 2001) and thus are easier to construct; moreover, they generally yield higher-affinity clones that have already undergone affinity maturation *in vivo*. Antibody selection from immunized phage libraries also possesses several advantages over the hybridoma method. Specifically, the screening process becomes much more efficient after enrichment of target-specific clones by biopanning, and the *in vitro* selection parameters such as temperature, binding time, wash stringency, and concentration can be controlled at will. Additionally, phage display technology is applicable to species for which suitable myeloma cell lines for hybridoma generation are not readily available, and the variable region genes of the antigen-specific clones can very easily be retrieved after the panning selection.

Despite these advantages, it is generally considered that a phage antibody library constructed from immunized sources is only useful against the antigen of immunization, and each new antigen requires the construction of a new immune library (Marks, 2004). This mode of thought is generally because an immune library in general is small in size and its repertoire is biased towards the immunizing antigen. However, the majority of B cells in an immunized repertoire is not specific for the antigen of immunization (Story et al., 2008), which suggests that a sufficiently large immune library might be a useful source of antibodies against non-immunizing antigens. In this work, 25 immune libraries constructed from about 50 rabbit spleens were combined into a library of > 10^{10} in size. The library was tested against a panel of non-immunizing antigens to evaluate the feasibility of using immune libraries against non-immunizing antigens.

MATERIALS AND METHODS

Library preparation

Phagemid DNAs of rabbit sub-libraries were provided by Young In Frontier (Korea). Electrocompetent cells of ER2537 *E. coli* strain were freshly prepared for each transformation as previously described (Rader et al., 2001). Several micrograms of phagemid DNA was mixed with 300 μl of electrocompetent cells prepared from a 100 ml culture and electroporated. Cells were rescued in SOC medium for 1 h at 37°C and then trans-

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Table 1. Immunizing antigen, size, grouping, and transformation titer of individual immune libraries used in this study.

Group	Antigen	Type	Size ($\times 10^8$)	Transformation titer ($\times 10^8$)
1	AFP	Native protein	3.3	
	AFP 1	Peptide	5.5	2.2
	AFP 2	Peptide	3.6	
2	PSA	Native protein	2.2	
	NSE 1	Peptide	4.6	140
	NSE 2	Peptide	1.2	
3	EGFR	Peptide	3.4	
	EGFR IP-1	Native protein	2.6	88
	EGFR IP-2	Native protein	3.8	
4	Akt	Peptide	8	
	Akt1	Peptide	8	
	Akt inclusion 1	Recombinant protein	3.5	50
	Akt inclusion 2	Recombinant protein	3.6	
5	PTP-SO3	Peptide	45	88
	GAPDH-SO3	Peptide	3.9	
6	pSTAT1	Peptide	1.5	19
	pSTAT5	Peptide	2.7	
7	pP38	Peptide	30	
	nP38	Peptide	20	
	rP38	Recombinant protein	40	51
	Erk1	Recombinant protein	60	
8	cMet-ECD-Fc 1	Fc fusion protein	40	
	cMet-ECD-Fc 2	Fc fusion protein	10	38
	cMet-ECD-Fc 3	Fc fusion protein	30	
	cMet-SEMA-Fc	Fc fusion protein	40	
	Total		380	500

AFP, alpha fetoprotein; PSA, prostate-specific antigen; NSE, neuron-specific enolase; EGFR, epidermal growth factor receptor; PTP, protein tyrosine phosphatase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

ferred to 400 ml of SB medium containing 100 μg/ml of ampicillin and 2% glucose (w/v). After overnight culture (12-16 h), cells were centrifuged and resuspended in 10 ml of SB medium. A half volume of 50% glycerol was subsequently added and thoroughly mixed, and 1 ml aliquots were frozen in liquid nitrogen and kept at -80°C. Phage libraries were rescued from the frozen *E. coli* stocks as previously described (Yang et al., 2009) and then combined into a single large rabbit scFv library.

Library panning and screening

Panning and ELISA screening against passively adsorbed antigens (proteins and protein-conjugated peptides/small molecules) were performed as previously described (Yang et al., 2009). Biotinylated peptide antigens were first captured on M-480 paramagnetic streptavidin-conjugated beads (Invitrogen) by mixing 50 μl of the beads with 1 μg of the peptide in 1 ml of PBS, followed by incubation for 15 min with gentle rotation. After washing twice with TBS-0.1% Tween20 (TBST), the beads were blocked in 3% skim milk-PBS for 1 h with rotation. Fifty microliters of magnetic beads without bound peptide was also blocked separately. After blocking, one library equivalent (10^{13} cfu) in 1 ml of 3% milk-PBS was added to the beads without peptide to deplete the library of streptavidin binders. After 1 h of depletion, the library was transferred to the peptide-bound beads and incubated at room temperature for 1 h with rotation. The beads were then washed (once for the first round, three times for the subsequent rounds) with TBST, and the bound phages were eluted with 1 ml of 100 mM triethylamine. Subsequent steps were performed as previously described (Yang et al., 2009). After four rounds of panning, ELISA screening was performed on the biotinylated peptide antigen captured by surface-coated avidin (10 μg/ml in PBS).

Table 2. Summary of library panning campaigns against various antigens. Only successful panning results are shown; panning against RANKL and several phosphopeptides (EGFR-pY845/pY1068/pY1114, PDFGR-pY740/pY1021) failed to produce target-specific antibodies.

Antigen	Selection rounds	ELISA positive/screened	Unique sequences/analyzed
EGFR-pY1173	4	4/94	2/4
EGFR-pY1148	4	3/192	1/3
P27-pY74	4	105/192	N/A
P27-pY88	4	1/192	N/A
actin	4	86/94	3/8
huVCAM-1	4	36/94	2/5
RSA	4	76/94	1/6
HEL	4	3/94	2/3
FITC	4	16/46	3/8

N/A, not analyzed.

Analysis of selected clones

Immunoblotting, immunoprecipitation, and ELISA analyses were performed by following standard protocols. For immunoblotting and ELISA experiments, purified scFv (Yang et al., 2009) or unpurified *E. coli* periplasmic extract containing scFv was used as a primary antibody. For immunoprecipitation of a target antigen in cell lysate, the scFv gene was cloned into a pcDNA3.1-based scFv-Fc expression vector. The scFv-Fc fusion protein was expressed from transiently transfected Freestyle® 293F cells (Invitrogen) by following the supplier's protocol and purified using protein G-agarose beads (Thermo Scientific).

RESULTS AND DISCUSSION

Twenty-five rabbit immune antibody libraries, all in scFv format (V_L - linker - V_H), were divided into eight groups based on the similarity of their antigens (Table 1). Phagemid DNAs of the libraries composing each group were combined and transformed into ER2537 *E. coli* strain by electroporation. The total transformation titer was 5×10^{10}, which may be sufficiently large for panning experiments but may not represent all of the diversity in the original libraries (combined size of 3.8×10^{10}). Phage was rescued from the eight transformed sub-libraries and mixed to yield the final rabbit immune antibody library. After four rounds of panning using the phage library, specific antibodies could be isolated against a majority of the protein antigens as well as some of the other antigens (Table 2). The types of antigens tested include proteins (RSA, lysozyme, RANKL, actin, hVCAM), phosphopeptides (pY845, pY1068, pY1114, pY1148, and pY1173 sequences of EGFR; pY74 and pY88 sequences of p27; and pY740 and pY1021 sequences of PDGFR), and a small molecule (fluoresceine). Panning was most successful with the protein antigens, against all of which ELISA-positive

A

ELVLTQSPSVQVNLGQTVSLTCTADTLSRSYASWYQQKPGQAPVLLIYRD
TSRPSGVPDRFSGSSSGNTATLTISGAQAGDEADYYCATSDGSGSSYQYV
FGGGTQLTVTGSSGGGGSGGGGGGSSRSSQSLEESGGRLVTPGTPLTLTC
TASGFSLSNYGMSWVRQAPGKGLEWIGYIWSGGRIYYANWAKGRFTISKT
SSTTVDLKMTSPTTEDTATYFCARDLYPNNIRAFNLWGQGTLVTVSS

B

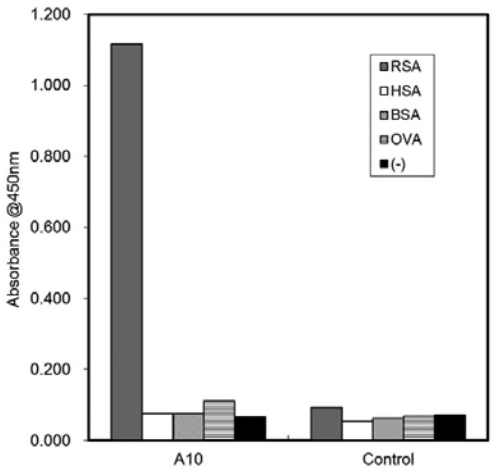

Fig. 1. (A) Translated sequence of the isolated rabbit anti-RSA scFv clone A10, in V_L - long linker - V_H format. The linker sequence is underlined. (B) Cross-reactivity ELISA of the RSA-specific clone A10. No cross-reactivity towards human serum albumin (HSA), bovine serum albumin (RSA), or ovalbumin (OVA, chicken egg white albumin) was detected. (-); Negative control (no antigen coated).

clones were identified except one (RANKL). A protein molecule offers a large surface area, many unique epitopes of various shapes, and a relatively rigid conformation, all of which favor facile selection of specific, high-affinity antibodies from a library. Notably, an antibody that is highly specific for rabbit serum albumin (RSA) was isolated from the library (Fig. 1A). This scFv contains a rabbit lambda light chain and a hypermutated variable heavy chain derived from rabbit *V_H1* (Crane et al., 1996; Knight and Winstead, 1997), confirming its leporine origin. This clone bound RSA with high specificity and without detectible cross-reactivity towards albumins of different species (Fig. 1B). Since self-reactive B cells are negatively selected during B cell development, the rabbit spleens used to construct the immune libraries are supposed to be depleted of B cells producing anti-RSA antibodies. It is likely that these scFv clones acquired their specificity towards RSA through chain shuffling (i.e. random recombination of light and heavy chains during scFv library construction) or incomplete clonal deletion of self-reactive B cell clones.

Only EGFR-pY1173 phosphopeptide yielded antibodies specific to phosphorylated EGFR; EGFR pY1148, p27-pY74, and p27-pY88 phosphopeptides yielded some clones that specifically bound to the phosphopeptide antigen in ELISA (Table 2), but none specifically detected the phosphoprotein in the immunoblot analysis (data not shown). Panning against the other phosphopeptides produced clones that were likely to bind phosphotyrosin (pTyr) regardless of the peptide sequence. Developing antibodies against protein phosphorylation using phosphopeptide antigens presents several difficulties due to the inherent nature of the antigen. Peptides have a small surface area and are flexible, which increases the entropic cost of the binding interaction, leading to lower binding affinity (Cobaugh et al., 2008; Zahnd et al., 2004). Further, antibodies produced against a peptide antigen do not necessarily bind a protein containing the peptide sequence. Additionally, phosphorylation-specific antibodies must discern a very small difference, i.e., a

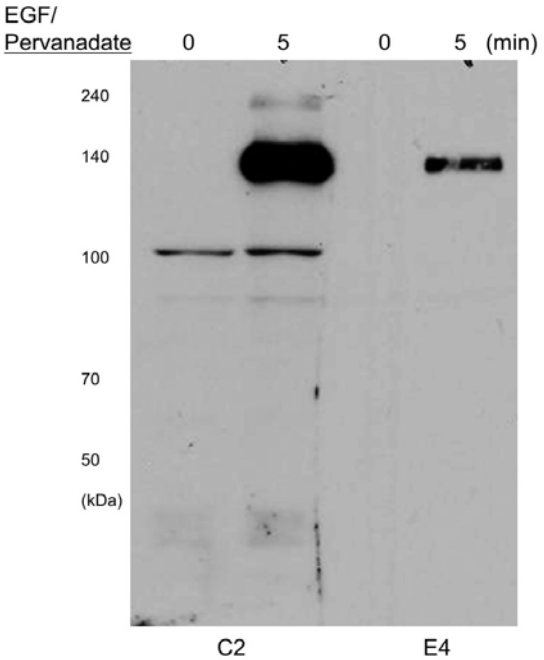

Fig. 2. Immunoblot analysis of two rabbit scFv clones (C2 and E4) against phospho-EGFR pY 1173. These scFvs specifically bound to EGFR in A431 cell lysate stimulated with EGF/pervanadate, whereas no binding was observed with serum-starved A431 lysate.

phosphate group. As a result of these factors, producing antibodies against a specific protein phosphorylation by phage display can be challenging and requires a high quality antibody library. In this study, we were able to isolate phosphorylation-

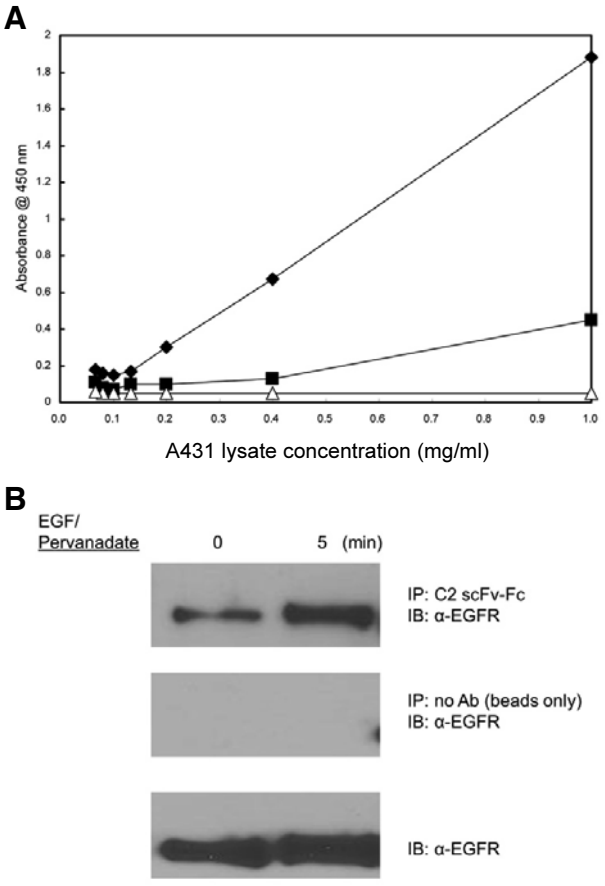

Fig. 3. (A) Sandwich ELISA detection of phospho-EGFR by a rabbit scFv clone C2. Purified scFv was coated on the plastic surface, A431 lysate (EGF/pervanadate treated or serum-starved) was added, and the binding was detected using an anti-EGFR antibody (cetuximab). Diamond, EGF/pervanadate-treated A431 lysate; square, serum-starved A431 lysate; triangle, PBS. (B) Immunoprecipitation of phosphorylated EGFR from A431 lysate by C2 anti-EGFR (pY1173) scFv-Fc fusion protein. non-specific binding of EGFR to protein G-agarose beads was not observed (middle). See text for details.

specific antibodies from the library using a synthetic phosphopeptide as an antigen. Although the combined library included immune libraries against EGFR (Table 1), it was confirmed that panning of these libraries did not yield any antibodies against EGFR-pY1173 (unpublished result). Therefore, it is likely that the anti-pY1173 antibodies were derived from the naïve portion of the library. Immunoblot analysis of the selected clones with EGF/ pervanadate-treated A431 cell lysate (Fig. 2) shows that the binding was highly specific for phosphorylated EGFR. The anti-pEGFR clone C2 also could detect undenatured phospho-EGFR in the cell lysate by sandwich ELISA and immunoprecipitation (Fig. 3). A low level binding of clone C2 to unstimulated A431 lysate was detectible in both experiments, possibly due to the basal level phosphorylation of EGFR.

In this work, a combined pool of rabbit immune scFv libraries was panned against various non-immunizing antigens. The results show that a sufficiently large immune library can, similar to a naïve library, yield specific antibodies against various non-immunizing antigens. This can be explained by the fact that, even after repeated immunization, only a portion of an animal's B-cell repertoire becomes reactive to the antigen; a larger portion remains unreactive and presumably naïve (Story et al., 2008). Thus, the ability of the immune system of a hyperimmunized animal to elicit humoral response to a second antigen (Gilden and Tokuda, 1963; Pleten, 2007) could be reproduced in the phage display system. These results demonstrate that a sufficiently large immune library can be a ready source of antibodies, not only against the immunizing antigen but also against many other non-immunizing antigens.

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REFERENCES

Barbas, C.F., 3rd, Kang, A.S., Lerner, R.A., and Benkovic, S.J. (1991). Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. Proc. Natl. Acad. Sci. USA *88*, 7978-7982.

Barbas, C.F., 3rd, Bain, J.D., Hoekstra, D.M., and Lerner, R.A. (1992). Semisynthetic combinatorial antibody libraries: a chemical solution to the diversity problem. Proc. Natl. Acad. Sci. USA *89*, 4457-4461.

Clackson, T., Hoogenboom, H.R., Griffiths, A.D., and Winter, G. (1991). Making antibody fragments using phage display libraries. Nature *352*, 624-628.

Cobaugh, C.W., Almagro, J.C., Pogson, M., Iverson, B., and Georgiou, G. (2008). Synthetic antibody libraries focused towards peptide ligands. J. Mol. Biol. *378*, 622-633.

Crane, M.A., Kingzette, M., and Knight, K.L. (1996). Evidence for limited B-lymphopoiesis in adult rabbits. J. Exp. Med. *183*, 2119-2121.

Gilden, R.V., and Tokuda, S. (1963). Antibody quality after sequential immunization with related antigens. Science *140*, 405-406.

Glanville, J., Zhai, W., Berka, J., Telman, D., Huerta, G., Mehta, G.R., Ni, I., Mei, L., Sundar, P.D., Day, G.M., et al. (2009). Precise determination of the diversity of a combinatorial antibody library gives insight into the human immunoglobulin repertoire. Proc. Natl. Acad. Sci. USA *106*, 20216-20221.

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., Allison, T.J., et al. (1994). Isolation of high affinity human antibodies directly from large synthetic repertoires. EMBO J. *13*, 3245-3260.

Huse, W.D., Sastry, L., Iverson, S.A., Kang, A.S., Alting-Mees, M., Burton, D.R., Benkovic, S.J., and Lerner, R.A. (1989). Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. Science *246*, 1275-1281.

Knight, K.L., and Winstead, C.R. (1997). Generation of antibody diversity in rabbits. Curr. Opin. Immunol. *9*, 228-232.

Marks, J.D. (2004). Monoclonal antibodies from display libraries. In Molecular Biology of B Cells, T. Honjo, F.W. Alt, and M. Neuberger, eds. (London, Elsevier Academic Press), pp. 511-531.

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

McCafferty, J., Griffiths, A.D., Winter, G., and Chiswell, D.J. (1990). Phage antibodies: filamentous phage displaying antibody variable domains. Nature *348*, 552-554.

Pleten, A.P. (2007). Production of different antibodies after simultaneous immunization of animals with two antigens. Bull. Exp. Biol. Med. *143*, 771-773.

Rader, C., Steinberger, P., and Barbas, C.F., III (2001). Selection from antibody libraries. In Phage Display: A Laboratory Manual, C.F. Barbas, III, D.R. Burton, J.K. Scott, and G.J. Silverman, eds. (Cold Spring Harbor, Cold Spring Harbor Laboratory Press), pp. 10.11-10.20.

Rothe, C., Urlinger, S., Lohning, C., Prassler, J., Stark, Y., Jager, U., Hubner, B., Bardroff, M., Pradel, I., Boss, M., et al. (2008). The human combinatorial antibody library HuCAL GOLD combines diversification of all six CDRs according to the natural immune system with a novel display method for efficient selection of high-affinity antibodies. J. Mol. Biol. *376*, 1182-1200.

Story, C.M., Papa, E., Hu, C.C., Ronan, J.L., Herlihy, K., Ploegh, H.L., and Love, J.C. (2008). Profiling antibody responses by multiparametric analysis of primary B cells. Proc. Natl. Acad. Sci. USA *105*, 17902-17907.

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

Yang, H.Y., Kang, K.J., Chung, J.E., and Shim, H. (2009). Construction of a large synthetic human scFv library with six diversified CDRs and high functional diversity. Mol. Cells *27*, 225-235.

Zahnd, C., Spinelli, S., Luginbuhl, B., Amstutz, P., Cambillau, C., and Pluckthun, A. (2004). Directed in vitro evolution and crystallographic analysis of a peptide-binding single chain antibody fragment (scFv) with low picomolar affinity. J. Biol. Chem. *279*, 18870-18877.