Biopanning of Antibody-Phage Clones Using Immunoplates Coated with Gel Slices of Electrophoresis: Immunogel-Biopanning

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Biopanning of a phage library using a Western blotting membrane is difficult because of high background binding. We propose a reliable biopanning method, namely, immunogel-biopanning, which is performed using immunoplates coated with a molecular species fractionated from a crude sample by native polyacrylamide gel electrophoresis (PAGE). The efficacy of this method was determined in model experiments using a human interleukin-18 (IL-18)-specific single chain Fv (scFv) phage clone.

Key words biopanning; antibody; phage library; native gel; ELISA

To establish a monoclonal antibody (mAb), a phage library does not require immunization, in contrast to cell-hybridization technology, which requires immunizing mice.[1,2] In a phage library, the antigen-specificity repertoire is not substantially limited because of the absence of biological negative clonal selection. This feature is particularly important for the development of antibody medicine, which often targets self-antigens. Among the revolutionary features of antibody-displaying phage libraries, several reports have suggested that a Western blotting membrane could be blotted with a phage library, resulting in the establishment of scFvs against new unknown molecules.[3,4] However, nonspecific binding of phage clones to membranes has hampered and restricted its availability in most cases. Furthermore, SDS-PAGE primarily resolved the denatured proteins, implying that it is hard to isolate the antibody clones specific to a conformation that is critical for biological function.

To overcome these problems, we attempted to establish an alternative approach, immunogel-biopanning, with less nonspecific binding. The two salient features of immunogel-biopanning are that the biopanning is performed using immunoplates coated with proteins eluted from sliced gels of electrophoresis and that the sensitivity to detect the target molecule can be highly elevated with biological amplification by using a phage clone as a probe.

In an experimental model using an established antigen-specific phage clone, we evaluated the feasibility and sensitivity of this method employing cell lysates mixed with a minute amount of IL-18. To achieve this, we employed a native PAGE for molecular separation and an anti-IL18 svFv-displaying phage clone, h18-108 as a probe that recognizes the receptor-binding-site 3 of native IL-18 but not denatured form.[5] Expecting a practical experimental condition, the amount of cell lysate was set as the highest dose of our preparation. We demonstrated that immunogel-biopanning was effective when cell lysates of 6.4 μg contained 50 ng of IL-18 and when the phage solution of 10^{11} cfu contained 10 cfu of target-specific phage clones. DNA sequencing of isolated phage clones after the fourth round of biopanning directly demonstrated that over 80% was the h18-108 clone.

Immunogel-biopanning is useful for the establishment of mAbs against new native conformations, such as prion protein and for the quantitative analysis of a given target molecule.

MATERIALS AND METHODS

Antibody-Phage Clone, Cell Lysate and Cytokine Native human IL-18-specific phage clone, h18-108 was employed.[5] The soluble h18-108 scFv has 50 nM of Kd. A murine neuroblastoma cell line, N2a was provided by S. Katamine (Nagasaki University Graduate School of Medical Science, Nagasaki). The cell lysates were prepared as described.[6] Recombinant IL-18 was purchased from MBL (Nagoya). The concentration of protein was determined using the D_C Protein Assay (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Electrophoresis Samples were not denatured by heating and SDS. Native PAGE (12.5%) was performed using running buffer without SDS as described.[5,7] Gel staining was carried out with a Silver Staining II kit (Wako, Osaka) or Coomassie Brilliant Blue (CBB: Nacalai Tesque, Kyoto).

Immobilization of Eluted Proteins onto Immunoplates Gels of experimental lanes were cut into several fractions. Each gel fraction was placed in 1.7 ml-tubes (Seiko, Fukuoka, Japan) punched with a 27G needle (TERUMO, Tokyo, Japan) and crushed by pushing them through the pore using the gasket of a 1 ml-syringe (TERUMO). Each gel fragment was incubated with phosphate-buffered saline (PBS) in immunoplates (F96 Maxisorp Nunc-Immuno Plate, Nunc, Roskilde, Denmark) at 4 °C. Ten hours later, the gel fragments were discarded, and the immunoplates were blocked with PBS containing 5% skim milk (Becton, Dickinson, Sparks, MD, U.S.A.). These immunoplates were used for immunogel-biopanning as well as the enzyme-linked immunosorbent assay (ELISA).

ELISA ELISA was performed as described.[5,8] Briefly, a phage solution (8×10^{10} cfu/40 μl/well) or anti-human IL-18 mAb (100 ng/40 μl/well, clone # 125-2H: MBL) was added to the protein-coated wells. The phage clone was detected using horseradish peroxidase (HRP)-conjugated anti-M13 mAb at a dilution of 1 : 1000 (Amersham Biosciences, Uppsala). Anti-human IL-18 mAb was detected with HRP-conjugated anti-mouse IgG at a dilution of 1 : 1000 (Jackson Im-

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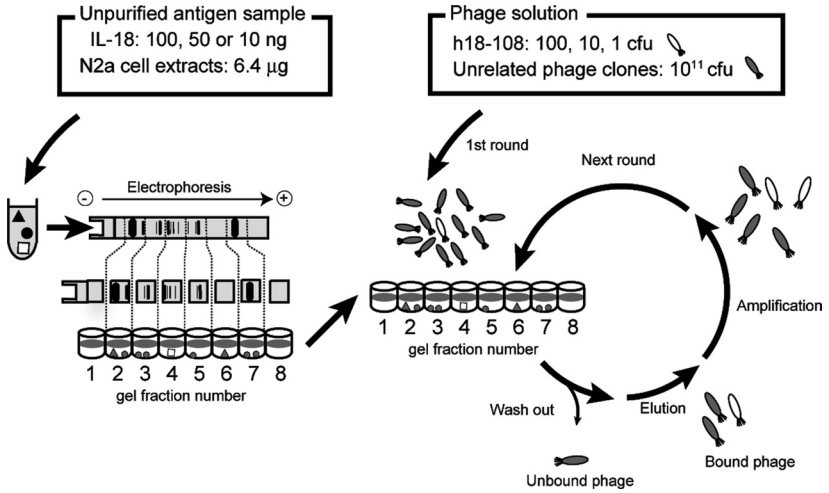

Fig. 1. Outline of the Immunogel-Biopanning

After samples (8 μl/lane) were separated by PAGE, running gels were fractionated by slicing gels. Each gel fragment was put into immunoplate wells, crushed to elute the proteins, and incubated overnight to coat the wells with the eluted proteins. Biopanning was performed in these wells.

munoResearch Lab., Inc., West Grove, PA, U.S.A.). After incubation with a 3,3′,5,5′-tetramethylbenzidine solution, the absorbance was measured at 450 nm.

Biopanning Biopanning was performed as described previously.[5,9] The model phage library was prepared by combining IL-18-nonbinding scFv-phage clones with a fixed number of h18-108 scFv-phage clones The unrelated phage clones stand for the mixture of IL-18-nonbinding scFv-phage clones. Briefly, the protein-coated wells were incubated with the phage solution (1×10^{11} cfu/300 μl/well). Bound phages were eluted by 0.1 M glycine-HCl (pH 2.2) and immediately neutralized with 1 M Tris–HCl (pH 9.1). The eluates were used for the measurement of the titer and the amplification of the recovered phage by infection with *E. coli*. TG-1 as described.[5] The amplified phages were used for the subsequent biopanning in the identical way to the first round.

DNA Sequencing The DNA sequence of the phages was determined using the Dye Terminator method as described.[5]

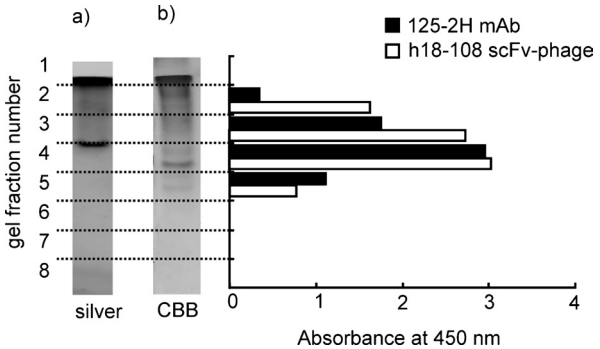

Fig. 2. Native-PAGE of Recombinant Human IL-18 Mixed with or without Cell Extracts

(a) Silver staining of the gel loaded with IL-18 alone. (b) Left panel: CBB staining of gel loaded with 8 μl of cell lysates (800 μg/ml) mixed with IL-18. The cell lysates were applied at the highest amounts of the prepared lysate. Right panel: Binding activity of 125-2H mAb and h18-108 scFv-phage to wells coated with IL-18 of eluates from each gel fraction as determined by ELISA.

RESULTS

Outline of Immunogel-Biopanning Crude samples are separated by native PAGE without the treatment of SDS and heating. After electrophoresis, running gels are fractionated by slicing. Each gel fragment is put into immunoplate wells, crushed to elute the proteins, and incubated overnight to coat the wells with the eluted proteins. Both biopanning and ELISA are performed using immunoplates treated with the same procedure. One of the duplicated lanes is stained with silver or CBB so that the running pattern can be visualized. The eluted phages are propagated as described under "Biopanning" in Materials and Methods. This procedure is repeated several times to amplify the sensitivity and focus the binding specificity. To determine the feasibility of this method, we tested the model system using a specificity-defined antibody phage clone.[5]

An IL-18-Specific scFv-Phage Clone, h18-108, Detects IL-18 Coated from Native PAGE Gel To determine the separation pattern of IL-18, IL-18 alone (100 ng/lane) was run on native PAGE. A discrete major band at fraction #3 or 4 was visible by silver staining (Fig. 2a). Under the identical condition, cell lysates mixed with 100 ng of IL-18 were resolved by native PAGE (Fig. 2b). Expecting a practical experimental condition, the amount of cell lysate was set as the highest dose (6.4 μg) of our preparation. The control lane was stained with CBB, while the gels of experimental lanes were sliced for fractionation and incubated in immunoplate wells. Although the resolved band of IL-18 was invisible by CBB staining, a murine anti-human IL-18 mAb, 125-2H, detected IL-18 in the four separated fractions, #2, 3, 4, and 5 in ELISA (Fig. 2b). No residual IL-18 was detected in wells coated with other fractions. Consistent with these results, the h18-108 scFv-phage clone showed identical ability to 125-2H for detecting IL-18, indicating that h18-108 definitely detects the trace of IL-18 under experimental conditions and also suggesting that the content in #2 and 5 appeared to be less than the detection level of silver staining.

Detection Sensitivity Related to the Contents of Target Protein Cell lysates (6.4 μg) mixed with varying amounts of IL-18 (100, 50, or 10 ng) were subjected to native PAGE. As described in Fig. 1, proteins in each gel fraction were

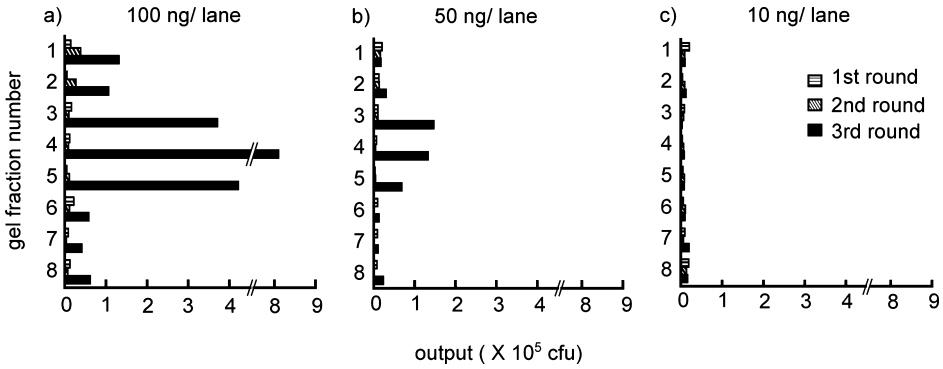

Fig. 3. Immunogel-Biopanning: Content of the Target Molecule in Crude Extracts

A cell extract mixed with varying amounts of IL-18 (a: 100 ng, b: 50 ng, or c: 10 ng/8 μl/lane) was subjected to native PAGE. Immunoplates were coated with proteins contained in each gel fraction followed by incubation with an scFv-phage solution (10^{11} cfu) containing 100 cfu of the h18-108 scFv-phage. The numbers of eluted phages were counted by phage titration.

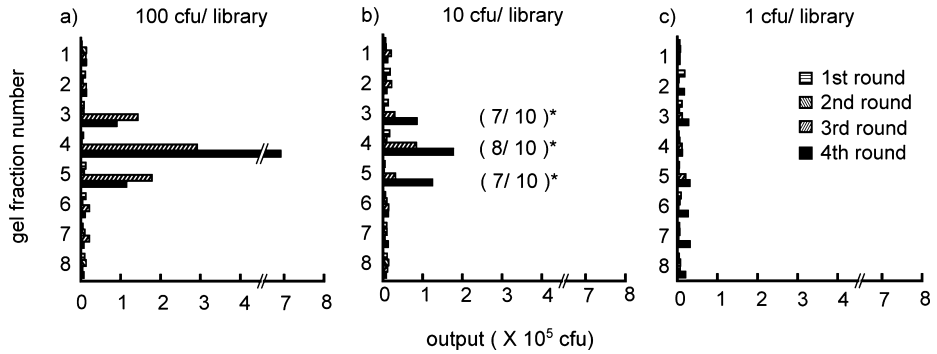

Fig. 4. Minimum Number of Target-Specific Phage Clones in a Phage Library

Cell lysates mixed with IL-18 (100 ng) were subjected to native PAGE. Immunoplates were coated with each gel fraction followed by incubation with an scFv-phage solution (10^{11} cfu) containing varying numbers of the h18-108 scFv-phage (a: 100 cfu, b: 10 cfu, or c: 1 cfu). The eluted phage was titrated and amplified. After the fourth round, the h18-108 clone was identified by DNA sequencing. The number in parentheses indicates the number of h18-108 clones among all clones tested (h18-108/ total tested).

coated to immunoplates. IL-18 coated in immunoplate wells was quantitatively estimated with ELISA using 125-2H mAb. When the cell lysates combined with 100 ng of IL-18 were resolved on PAGE, 2, 27, 38, or 5 ng of IL-18 was detected at the gel fractions #2, 3, 4, or 5, respectively. In the case of 50 ng of IL-18, the amount of the coated IL-18 was almost 1/10 of that in the case of 100 ng of IL-18. IL-18 was not detected in 10 ng under this procedure. Biopanning was performed using these immunoplates (Fig. 3). In the first round, the protein-coated wells were incubated with 10^{11} cfu of unrelated phage clones containing 100 cfu of the h18-108 scFv-phage. The amplified phages from each well were panned to the same duplicated well at subsequent rounds. The numbers of eluted phages on each round were calculated by phage titration. After the third round, a significant number of phages were recovered at gel fractions #3, 4, and 5 in the case of 100 ng (a) or 50 ng (b) of IL-18 but not at other gel fractions. In the case of 10 ng of IL-18, no increase in the number of phages was attained at any gel fraction. These patterns were consistent with the results shown in Fig. 2, indicating that this method definitely works if the crude extracts contain 50 ng of the target protein at the beginning of native PAGE.

Minimum Number of Target-Specific Phage Clones in a Phage Library The requirement of the minimum number of target-specific phage clones was estimated for the availability of a phage library to perform immunogel-biopanning. Cell lysates (6.4 μg) mixed with 100 ng of IL-18 were subjected to native PAGE and coated to immunoplates as described (Fig. 1). The phage solution was prepared by mixing a limited number of h18-108 scFv-phage clones with 10^{11} cfu of unrelated phage clones and put into each protein-coated well. In the third round of biopanning, a significant increase in the number of recovered phages was observed at #3, 4, and 5 gel fractions when the phage solution contained 100 cfu or 10 cfu of the h18-108 scFv-phage (Figs. 4a, b). The fourth round produced significantly amplified results.

Efficacy of Immunogel-Biopanning The phage solution contains a limited number of h18-108 scFv-phage clones with 10^{11} cfu of unrelated phage clones. The applied protein sample contains a minute amount of target protein in the presence of a large amount of unrelated proteins of cell lysates. Therefore, it is important whether or not the amplified results faithfully reflect the specific amplification to confirm the feasibility of this method. To verify the specificity of this amplification, the randomly selected phage clones were directly sequenced on these scFv genes. The results demonstrated that over 80% were the h18-108 clone (data presented in parentheses marked with * of Fig. 4b). This result indicated the reliability of this method, immunogel-biopanning. The salient feature is that there is no increase in binding of phages for the rest of the gel fractions, even after the fourth round, indicating the fine specificity for this detection. This result indicates that non-specific binding of phages as

pointed out in the membrane-blotting method does not hamper the immunogel-biopanning method. In the case of one cfu of the h18-108 scFv-phage in the phage solution (Fig. 4c), the enrichment was not shown even in the fourth round of biopanning. This might be attributed to non-specific loss due to the extreme case of 1 cfu of a specific clone.

DISCUSSION

Filamentous phages attach non-specifically to a Western blotting membrane, making it difficult to enrich antigen-specific phage antibodies from phage libraries.[3,4] Liu *et al.* reported that methanol-treatment of the polyvinylidene fluoride (PVDF) membrane could overcome this difficulty.[3] However, there have been few reports of success in the discovery of new protein molecules using this method.

In our hands using IL-18, Liu's protocol gave 1 to 2×10^6 phage-recovery (background) after the phage-amplification eluted from 25 mm^2 PVDF membrane coated with or without IL-18, even if the library contained no IL-18-specific phage clones. Therefore, we did not perform the experiments to compare quantitatively to Liu's method using purified IL-18 or its mixture with unrelated proteins. The purpose of this study was, rather, to develop the panning procedure with far less non-specific phage-binding and determine the minimum amount of target molecule in a sample that may result from a total loss occurring after experimental manipulations, such as electrophoresis, gel slicing, elution efficiency, protein coating, or sensitivity of ELISA.

To simulate the practical experimental conditions, the IL-18 was mixed with the cell lysates and analyzed for feasibility. The cell lysates contained 6.4 μg/8 μl/lane which was the highest amount of sample applied in our experiments. We showed first that immunogel-biopanning successfully worked when the cell lysates contained 50 ng of the target molecule. The amount of 50 ng can be reduced if an experimental apparatus or a manipulation method is devised. Secondly, this method works when the phage solution contains 10 cfu of target-specific phage clones in a total of 10^{11} cfu of an unrelated phage population. This frequency of a target-specific clone is quite usual in phage libraries. As the unrelated phages were a mixture of several clones of non-IL-18 binding phage, therefore, this result was attributed to the specific clone selection *via* anti-IL-18 scFv displaying on a phage surface but not the non-specific binding *via* non-scFv phage surface molecules. This result indicated that 10 out of 10^{11} phages were able to detect 5 ng of the target protein per well by this procedure (Figs. 3, 4). It is noteworthy that a target protein that is invisible by even silver staining can be accurately detected by antibody-phage propagation. It is also of interest that the amplification of the detection sensitivity is accomplished by phage propagation, while in other methods it is usually attained by enzymatic chemical reactions or electrical manipulations.

Another salient feature of our experiment is that this method was attained using IL-18 conformation-specific scFv phage clones. Biopanning using Western blotting is not suitable for isolation of conformation-specific phage clones. It is well known that the conformation of protein is critically related to severe pathogenesis such as in amyloid β or prion proteins.[10,11] In these cases, the antibodies specific to various conformers are promising tools for analyzing the pathogenesis of protein conformation diseases and development of immunotherapeutic reagents. The immunogel-biopanning may be useful for this purpose. We are isolating scFv-phage clones specific to a distinctive conformer, Barghorn's globulomer of amyloid $\beta_{1—42}$,[12] from the mixture of various conformers, employing this immunogel-biopanning method [in preparation].

Thus, immunogel-biopanning is useful for the isolation of a monoclonal antibody specific to a rare target molecule using crude mixtures of tissue or cell lysates. This method may also be promising for quantitative purposes and have much higher sensitivity if it is combined with the recently reported immuno-polymerase chain reaction method.[13]

Acknowledgements Preliminary experiments were performed by H. Kokuryou (EIKEN CHEMICAL CO., LTD., Tokyo). This research was partially supported by Core Research for Evolutional Science and Technology (CREST) of the Japan Science and Technology Corporation.

REFERENCES

1) Smith G. P., *Science*, **228**, 1315—1317 (1985).
2) Clackson T., Hoogenboom H. R., Griffiths A. D., Winter G., *Nature* (London), **352**, 624—628 (1991).
3) Liu B., Huang L., Sihlbom C., Burlingame A., Marks J. D., *J. Mol. Biol.*, **315**, 1063—1073 (2002).
4) Furuta M., Ito T., Eguchi C., Tanaka T., Wakabayashi-Takai E., Kaneko K., *J. Biochem.* (Tokyo), **132**, 245—251 (2002).
5) Hamasaki T., Hashiguchi S., Ito Y., Kato Z., Nakanishi K., Nakashima T., Sugimura K., *J. Biochem.* (Tokyo), **138**, 433—442 (2005).
6) Nishida N., Katamine S., Manuelidis L., *Science*, **310**, 493—496 (2005).
7) Fukumoto T., Torigoe N., Kawabata S., Murakami M., Uede T., Nishi T., Ito Y., Sugimura K., *Nat. Biotechnol.*, **16**, 267—270 (1998).
8) Kaji M., Ikari M., Hashiguchi S., Ito Y., Matsumoto R., Yoshimura T., Kuratsu J., Sugimura K., *J. Biochem.* (Tokyo), **129**, 577—583 (2001).
9) Hashiguchi S., Nakashima T., Nitani A., Yoshihara T., Yoshinaga K., Ito Y., Maeda Y., Sugimura K., *J. Biochem.* (Tokyo), **133**, 43—49 (2003).
10) Weiner H. L., Frenkel D., *Nat. Rev. Immunol.*, **6**, 404—416 (2006).
11) White A. R., Enever P., Tayebi M., Mushens R., Linehan J., Brandner S., Anstee D., Collinge J., Hawke S., *Nature* (London), **422**, 80—83 (2003).
12) Barghorn S., Nimmrich V., Striebinger A., Krantz C., Keller P., Janson B., Bahr M., Schmidt M., Bitner R. S., Harlan J., Barlow E., Ebert U., Hillen H., *J. Neurochem.*, **95**, 834—847 (2005).
13) Guo Y. C., Zhou Y. F., Zhang X. E., Zhang Z. P., Qiao Y. M., Bi L. J., Wen J. K., Liang M. F., Zhang J. B., *Nucleic Acids Res.*, **34**, e62 (2006).