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METHODS AND PROTOCOLS

Enrichment of *Escherichia coli* spheroplasts displaying scFv antibodies specific for antigens expressed on the human cell surface

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Abstract Anchored periplasmic expression (APEx) is a method for isolating high affinity ligand-binding proteins from large combinatorial libraries, and antibodies highly specific for soluble antigens were successfully isolated from APEx antibody libraries in combination with flow cytometric sorting (Harvey et al., Proc Natl Acad Sci USA 101(25):9193–9198, 2004). However, many disease markers and drug targets are localized on the cell surface, and often, unique posttranslational modifications and/or properly folded epitopes are lost when they were expressed and isolated in soluble form. In this study, we demonstrate that *Escherichia coli* spheroplasts, displaying antibodies and screened by a combination of plate-panning and flow cytometric sorting, can be used for isolating antibodies specific for antigens on the human cell surface. Two rounds of plate-panning followed by one round of flow cytometric sorting resulted in 7,200-fold enrichment of antibodies specific for the protective antigen of *Bacillus anthracis* from a large excess of spheroplasts expressing a scFv antibody to digoxin (a negative control). There is the potential to use this technique for library screening to find novel antibodies against disease cell surface antigens.

Keywords Anchored periplasmic expression · Spheroplasts · scFv · Antigens on the human cell surface

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Introduction

Protein display technologies such as phage, bacteria, and yeast display have been developed for isolating antibodies from large libraries of recombinant antibodies and have become major tools for generating monoclonal antibodies for research and the clinic (Hoogenboom 2005). Phage display antibody libraries can be readily screened for binding to antigens presented on whole cells by "panning" (Hoogenboom et al. 1991; Du et al. 2006). On the other hand, yeast and bacterial surface displays have been mainly employed to screen antibodies for soluble antigens (Boder et al. 2000; Daugherty et al. 1999; Boder and Wittrup 1997; Georgiou et al. 1997). However, many disease markers and drug targets are localized on the cell surface, and lots of them require unique posttranslational modifications and/or properly folded epitopes (Binyamin 2006; Linenberger et al. 2002). Therefore, direct screening of antibodies against cells is of great interest for many applications. Recently, yeast display was successfully used to isolate novel antibodies that target the blood–brain barrier by panning against intact rat brain endothelial cell line (Wang et al. 2007; Wang and Shusta 2005). On the other hand, bacterial display has not been used

to screen antibody libraries for binding onto intact human cells. Towards this end, anchored periplasmic expression (APEx; Harvey et al. 2004), an *Escherichia coli* display system employing proteins tethered to the periplasmic side of the inner membrane, recently showed the ability of binding to immobilized ligands on beads (Jung et al. 2007). Compared to yeast display systems, APEx and other bacterial display methods offer the advantage of much large library size, resulting from the higher transformation efficiency of *E. coli* and faster growth for accelerating the screening process. For these reasons, the use of APEx-displayed scFv and IgG libraries (Ge et al. 2010; Mazor et al. 2008) is likely to facilitate and expedite the isolation of antibodies to cell surface proteins.

In this study, we show that antibody fragments displayed on *E. coli* spheroplasts can specifically recognize an antigen on the surface of either adherent or suspension human cells. As a model system, we employed the M18 scFv, which binds to the protective antigen domain 4 (PAD4) of *Bacillus anthracis* with high affinity (Harvey et al. 2004) and, as an unrelated control, the 26-10 scFv, which binds to cardiac glycoside digoxin and related molecules such as digoxigenin (Francisco et al. 1993). We have developed protocols for the enrichment of *E. coli* spheroplasts on human H1299 cells expressing the PAD4 antigen by a combination of panning followed by flow cytometric sorting. Using this approach, bacterial spheroplasts expressing the anti-PAD4 scFv could be readily enriched from a large excess of spheroplasts displaying control antibodies. The technique can be applied to screen antibodies specific for native antigens located on intact cell surface.

Materials and methods

Construction of vectors

The gene encoding the PAD4 sequence (refer to Electronic supplementary material) was synthesized by overlap polymerase chain reaction (PCR) using the primer set in the Electronic supplementary material. Primers were designed using DNAWorks (http://mcl1.ncifcrf.gov/dnaworks/dnaworks2.html). The PAD4 gene was cloned into *Bgl*II–*Sal*I-digested pDisplay plasmid (Invitrogen) to yield pDisplay-PAD4. PAD4 can be expressed on the surface of human cells via fusion to the transmembrane domain of the human platelet-derived growth factor receptor (PDGFR; Gronwald et al. 1988) at the C-terminal in the pUHD10-3-display vector (Gossen and Bujard 1992). For this purpose, the DNA sequence in pDisplay (Invitrogen) including the Ig κ-chain leader sequence, HA epitope tag, multiple cloning region, c-myc epitope tag, and PDGFR transmembrane domain was amplified using the primer pair 5′-ACGCGAATTCGCCAC CATGGAGACAGACACACTCCTGCT and 3′-AAC TAGTCTAGAC TAACGTGGCTTCTTCTGCCAAAG CAT, cut with *Eco*RI and *Xba*I and subcloned into the downstream of tet response promoter between *Eco*RI and *Xba*I sites to yield pUHD10-3-display. Then, PAD4 was cloned into pUHD10-3-display between *Bgl*II and *Sal*I sites to yield pUHD10-3-display-PAD4.

Bacteria cultures

All studies were carried out using *E. coli* Jude-1 cells (DH10B (Invitrogen) harboring the F′ factor derived from XL1-blue (Stratagene) was prepared by Hayhurst, A., Iverson, B.L., and Georgiou, G.; Kawarasaki et al. 2003). Jude-1 cells carrying pBAD30-KmR-GFPmut2 and either pAK200-2610 or pAK200-M18 were grown at 37 °C in Terrific Broth (TB) supplemented with 2% (*w*/*v*) glucose, chloramphenicol (40 μg/ml), and kanamycin (50 μg/ml). pBAD30-KmR-GFPmut2, pAK200-2610, and pAK200-M18 were used to express green fluorescent protein gene (GFP), 26-10, and M18 scFv, respectively (Jung et al. 2007). After overnight growth, the cells were diluted 1:100 in fresh TB medium without glucose and incubated at 37 °C to about 0.5 OD_{600}. Then, the cells were transferred to 16 °C, and 1 mM of IPTG (isopropyl-1-thio-b-D galactopyranoside) and 0.2% (*w*/*v*) arabinose were added to induce the expression of antibodies and GFP, respectively.

Preparation of spheroplasts

After induction for 18 h, cells (equivalent to 1 ml of 4.5 OD_{600}) were collected by centrifugation, washed twice in 1 ml of 10 mM Tris–HCl (pH 8.0), and resuspended in 1 ml of STE solution (0.5 M sucrose, 10 mM Tris–HCl, and 10 mM EDTA, pH 8.0). After incubation with rotating-mixing at 37 °C for 30 min, the cells were pelleted by centrifugation at 12,000×*g* for 1 min and washed in 1 ml of solution A (0.5 M sucrose, 20 mM $MgCl_2$, 10 mM MOPS, pH 6.8). The pellet was resuspended in 1 ml of solution A containing 1 mg/ml of hen egg lysozyme and incubated at 37 °C for 15 min. The resulting suspension was centrifuged at 12,000×*g* for 1 min, and the spheroplasts were resuspended in 0.5 ml of optimal-MEM (Invitrogen) plus 1% bovine serum albumin (BSA).

PAD4-displaying human cells

Human non-small cell lung carcinoma H1299 cells were maintained at 37 °C and 5% CO_2, in DMEM containing 10% fetal bovine serum. H1299 cells were used to display PAD4 transiently or in an inducible manner. For transient display, pDisplay-PAD4 was transfected into H1299 cells using the Lipofectamine 2000 kit (Invitrogen), and the cells

were used for binding spheroplasts 24 h after transfection. To establish an inducible PAD4-displaying human cell line, H1299 cells were co-transfected with pTet-on (Clontech) and pUHD10-3-display-PAD4 DNA. In the following day, the cell culture was split into two, and the cells were maintained in 450 μg/ml G418 (also known as Geneticin, Amresco) for 2 weeks to selectively kill negatively transfected cells. The G418-resistant cells were plated at a density of 5×10^5 cells per 10-cm dish, and doxycycline at the final concentration of 2 μg/ml was added to induce the PAD4 expression for 48 h. The protein contains a c-myc epitope tag at the C-terminal of PAD4, and the c-myc epitope tag was originally from the pDisplay plasmid. The cells were harvested, probed with 1:400 dilution of anti-c-myc mAb (9E10, Calbiochem) in optimal-MEM (Invitrogen) for 30 min at 4 °C. Then, the cells were washed with PBS and incubated with 1:300 dilution of fluorescein isothiocyanate (FITC)-labeled rabbit anti-mouse FITC (Jackson ImmunoResearch) for 30 min at 4 °C. After washing, the cells were suspended in DMEM medium. The cells with the ~5% highest FITC fluorescence were sorted by BD FACSAria II (Becton Dickinson). After three rounds of sorting, the cells (dubbed H1299-tet on-PAD4) were established with 63% cells displaying PAD4 on the cell surface.

Spheroplast panning and sorting

H1299-tet on-PAD4 cells were seeded onto 10-cm dishes at a density of 5×10^5 cells/dish and incubated with 2 μg/ml doxycycline. After 60 h of induction, the dishes were washed with PBS, and then blocked with optimal-MEM plus 1% BSA at room temperature for 20 min. About 80 OD_{600} (20 mL of 4 OD_{600}/ml) of well mixed *E. coli* spheroplasts resuspended in optimal-MEM were pipetted onto the plate. The plates containing spheroplasts and H1299-tet on-PAD4 cells were incubated at 37 °C for 1 h. After incubation, the dishes were washed four times with PBS to remove the non-binding spheroplasts. After washing, the human cells–spheroplast mixture was trypsinized off the plate. Plasmid DNA was isolated from the mixture and then transformed into electro-competent *E. coli* Jude-1 cells for the next round of panning or into the *E. coli* Jude-1 carrying GFPmut2 for FACS (Fluorescence Activating Cell Sorter) sorting.

For the FACS analysis or sorting, the induced H1299-tet on-PAD4 cells were trypsinized off the plate and resuspended in the optimal-MEM containing 1% BSA. In Eppendorf tubes, 10^6 cells were incubated with 2 OD_{600} spheroplasts for 1 h at 37 °C with shaking and washed in 1 ml PBS to remove unbound spheroplasts by centrifugation at 300×*g* for 2 min. By gating on the populations displaying the distinctive high forward scattering and high side scattering containing human cells with or without spheroplasts, the fluorescence level from GFPmut2-expressing spheroplasts bound to the gated human cells was analyzed using flow cytometry on a FACSCalibur instrument (Becton Dickinson). The GFPmut2 was excited by a 488-nm laser, and the emitted fluorescence at 530/30 nm was detected. We used BD FACSAria II (Becton Dickinson) to sort human cell-bound spheroplasts with the same set of 488 nm laser-line and emission fluorescence 530/30 nm.

At the early stage of this study, H1299 cells transiently transfected with pDisplay-PAD4 were used for spheroplasts panning and sorting, and results similar to those using H1299-tet on-PAD4 cells were obtained.

Spheroplasts enrichment onto H1299-tet on-PAD4 cells

Spheroplasts displaying the M18 scFv and the 26-10 scFv were prepared and mixed at 1:100, 1:1,000, or 1:10,000 ratios. The mixture was enriched by panning with H1299-tet on-PAD4 cells. The plasmid coding scFv genes was isolated from spheroplasts bound on H1299-tet on-PAD4 cells and transformed into the electro-competent *E. coli* Jude-1 cells. To determine panning efficiency, the number of M18 scFv-expressing clones was evaluated by colony PCR from colonies picked at random using M18 scFv specific primers (5′-ATATGCTAGCGATATTCAGATGACACAGACT and 3′-GCGTTTGCCATCTTTTCATAATCAAAATCACC). After one (at 1:1,000) or two (at $1:10^4$) rounds of panning, plasmid DNA was transformed into the *E. coli* Jude-1 cells expressing GFPmut2 for FACS sorting. H1299-tet on-PAD4 cells rendered fluorescent by the binding of GFP-expressing spheroplasts were sorted using BD FACSAria II (Becton Dickinson). The scFv plasmids were recovered, and the percentage of clones encoding the M18 scFv was determined by colony PCR.

Results

Binding of spheroplasts to H1299-PAD4 cells

In order to test if spheroplast-tethered antibodies can specifically bind to the PAD4 protein displayed on the human cell surface, we transiently transfected human H1299 cells with pDisplay-PAD4. The ability of spheroplasts displaying the anti-protective antigen M18 scFv antibody fragment to bind to adherent PAD4-expressing H1299 cells was investigated. Spheroplasts co-expressing GFP were used so that binding onto the surface of adherent H1299-PAD4 could be directly observed by light microscopy. As can be seen in Fig. 1, the M18-scFv expressing spheroplasts bind selectively onto H1299-PAD4 but not onto H1299 cells that do not express antigen. In control experiments, GFP encoding spheroplasts also expressing the anti-digoxin 26-10 scFv did not bind on either H1299-PAD4 or H1299 cells.

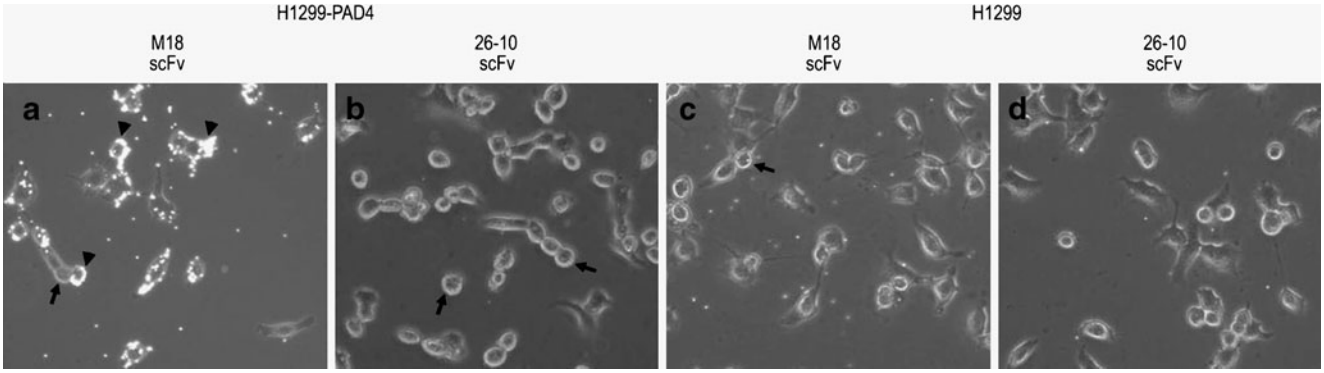

Fig. 1 Fluorescence images showing the binding of spheroplasts displaying M18 scFv to H1299-PAD4 cells. Representative fluorescence micrographs of spheroplasts M18 scFv binding to (**a**) H1299-PAD4 and (**c**) H1299, and 26-10 scFv binding to (**b**) H1299-PAD4 and (**d**) H1299. The *arrowheads* point to the spheroplasts that contain GFP, and the *arrows* point to autofluorescence from the edges of human cells

To simplify subsequent experiments, we generated human H1299 cells expressing the PAD4 antigen under the control of the doxycycline-inducible promoter. For this purpose, following selection, PAD4-displaying H1299 cells were enriched by three rounds of FACS. An H1299-tet on-PAD4 cell population under the doxycycline control was established with 63% cells displaying PAD4 on the surface (Fig. 2a). *E. coli* spheroplasts expressing the M18-scFv were shown to specifically bind to these cells in a manner similar to the results shown in Fig. 1 (data not shown).

In another strategy, spheroplasts were mixed with suspended H1299-tet on-PAD4 cells following trypsinization. The H1299-tet on-PAD4 cells were incubated with *E. coli* spheroplasts for 1 h in optimal-MEM medium, washed

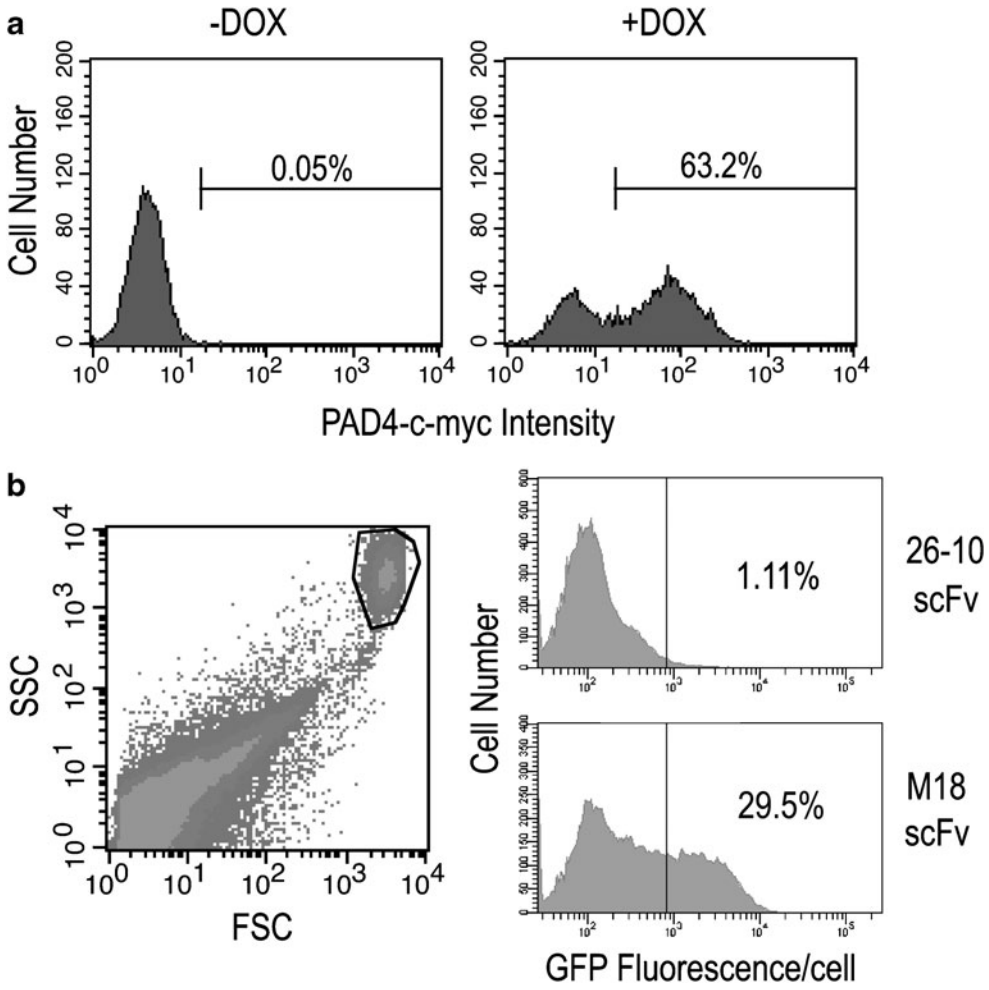

Fig. 2 FACS analysis of spheroplasts with H1299-tet on-PAD4. **a** Detection of PAD4 displaying on the surface of H1299. The PAD4 level is detected using anti-c-myc tag antibody in the absence (−Dox) or presence (+Dox). **b** Selection of highly fluorescent spheroplast-bound cells using FACS by gating the region exhibiting distinct scatterings (forward scattering and side scattering). Histograms for H1299-tet on-PAD4 incubated with spheroplasts displaying 26-10 scFv and M18 scFv

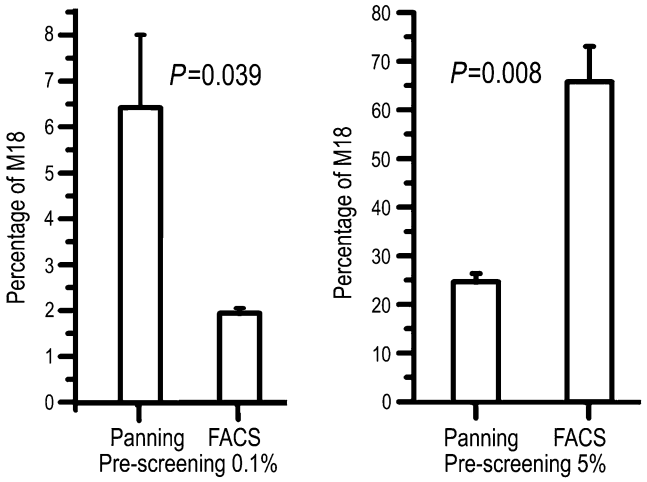

Fig. 3 Effect of panning or FACS sorting on enrichment efficiency at low and high initial M18 purity. Mixtures of spheroplasts at the indicated M18 purity were panned or sorted against H1299-tet on-PAD4 cells. M18 percentage was determined by colony PCR after each round. Mean±SD of three independent experiments is shown; $P<0.05$; Student's t test, comparison of panning, and FACS

in PBS once, and the binding to spheroplasts expressing either specific (M18 scFv) or control (26-10 scFv) antibody fragments was evaluated by FACS. As seen in Fig. 2b, H1299-tet on-PAD4 mixed with M18 spheroplasts exhibited significantly increased green fluorescence, enabling easy discrimination from the H1299-tet on-PAD4 cells that had been mixed with spheroplasts expressing the 26-10 control scFv. This result confirms that spheroplasts expressing M18 scFv can specifically bind PAD4 displayed on H1299 human cells, the specific binding in the suspended form should be able to enrich spheroplasts expressing M18 scFv by flow cytometry sorting.

Enrichment of M18 scFv displaying spheroplasts

The results above suggest that spheroplasts expressing the M18 scFv can bind to H1299-tet on-PAD4 both in adherent and suspension states, and therefore open up alternative strategies for optimization of the experimental conditions required for the facile enrichment of spheroplasts expressing antibodies specific to cell surface antigens. To simulate library-screening experiments, we examined whether M18 scFv expressing spheroplasts could be enriched from a large excess of spheroplasts displaying control scFv by panning. Cells expressing the M18 scFv were mixed with a 100-fold excess of spheroplasted *E. coli* 26-10 scFv, and after one round of panning onto H1299-tet on-PAD4 cells, plasmids were recovered and transformed into *E. coli* Jude-1. Following plating onto selective medium, colony PCR revealed that ten of 41 clones contained M18 scFv, indicating a 24.4-fold enrichment in a single round.

With a 1:1,000 mixture of M18 scFv and 26-10 scFv cells, one round of panning yielded a 64±16-fold enrichment of the M18 scFv encoding cells to 6.4±1.6% M18 as determined by colony PCR. Enrichment by FACS sorting instead of panning yielded a somewhat lower enrichment (20±1.2-fold enrichment to 2.0±0.1% M18 population on average). On the other hand, we observed that when the percentage of positives in a mixture is higher (5% or a 1:20 ratio), panning resulted in enrichment from 5% to 24.6±1.7%, while one round of FACS sorting yielded an *E. coli* population in which 65.6±7.3% of the bacteria expressed the M18 scFv. These results indicate that panning is more effective at low initial purity (≤1:1,000), whereas at a higher initial purity (i.e., ~5%), FACS sorting shows a better effect on enrichment (Fig. 3).

It should be noted that at purity lower than 1:1,000, panning harvests sufficient amount of spheroplasts and thus sufficient plasmid DNA for direct transformation of bacteria for next round of enrichment, while FACS sorting does not yield enough plasmid DNA, and PCR rescue of the insert is needed. Based on these results, we adapted a two-step enrichment procedure with panning used first to enrich the binder spheroplasts followed by plasmid isolation and transformation into Jude-1/GFPmut2 electro-competent cells for FACS sorting. In addition to final enrichment, flow cytometry also provides a quantitative means for determining the apparent affinity of library clones binding to the target cells.

Using this procedure, with a 1:1,000 mixture, in one experiment, the first round of panning yielded a 51-fold enrichment to 5.1% of bacteria encoding the M18 scFv. Plasmids recovered from the panning step were transformed into Jude-1/GFPmut2 and sorted. Colony PCR revealed that 65.6% clones of the clones in the sort solution contained the M18 scFv gene, resulting in a 656-fold overall enrichment (Fig. 4).

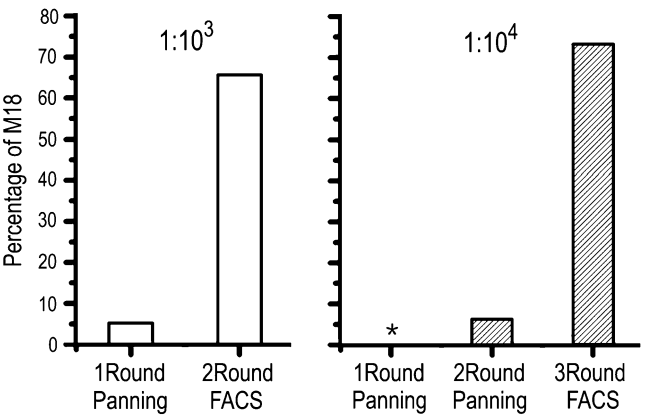

Fig. 4 Multiple-round enrichment of M18 spheroplasts from a background of irrelevant 26-10 spheroplasts. Mixtures of spheroplasts at the indicated M18/26-10 ratios were panned and FACS sorted against H1299-tet on-PAD4 cells. M18 percentage was determined by colony PCR after each round (*asterisks* means not detected)

Finally, the enrichment efficiency at an initial purity 1:10^4 was determined. Two rounds of panning resulted in a population containing 6.25% M18 scFv expressing cells. After a round of FACS sorting, the sort suspension contained 72.9% M18 scFv encoding bacteria indicating a 7,290-fold overall enrichment after two pannings and one FACS sorting (Fig. 4).

Discussion

Many disease markers and drug targets are localized on the cell surface and often consist of proteins that are postranslationally modified, particularly by extensive glycosylation (Ohtsubo and Marth 2006). Loss of glycosylation affects the efficiency of folding and stability (Shental-Bechor and Levy 2008, 2009). Altered glycosylation is considered a universal feature of cancer cells (Kim and Varki 1997; Nita-Lazar et al. 2009; Brockhausen 2006). Screening antibodies against intact cells has the potential to obtain antibodies specific for native antigens which have complex posttranslational modifications even when the identity of these antigens is unknown. This work demonstrates the potential of bacterial (spheroplast) display for selections on human cells. We employed Anchored Periplasmic Display or APEx with plate panning and flow cytometric sorting to show that rare cell surface binding antibodies can be isolated from a large excess of spheroplasts displaying antibodies of unrelated antigen specificity. We found that two rounds of plate panning followed by one round of flow cytometric screening resulted in over 7,290× enrichment.

This approach combines the advantages of conventional panning and of quantitative cell sorting via FACS. As can be seen in Fig. 3, the efficiency of FACS is poor at low initial binder purity (≤1:1,000). A similar result was reported for the binding of spheroplasts on immobilized antigen on beads (Jung et al. 2007). We believe that this is a consequence of the relative ratio of target cells to antibody displaying spheroplasts. When the percentage of binding spheroplasts is low, the "dilution of the spheroplasts" with an excess of antigen expressing human cells results in lower cell fluorescence that in turn reduces the sorting efficiency. For this reason, it is preferable to first carry out enrichment by panning to recover of the binding spheroplasts to increase the binder percentage. When the number of binding spheroplasts is sufficiently high, fluorescent labeling of the cells is more efficient. Under these conditions, the quantitative nature of FACS sorting constitutes a distinct advantage.

There is the potential to use this technique for library screening to find novel antibodies against disease cell surface antigens. Bacterial display enables us to construct very big combinatorial library (size >10^9). This new APEx-based screening technique is expected to prove very useful for isolating new antibodies for disease diagnostics and therapeutic targets from combinatorial libraries. Recently, full-length IgG antibodies have been displayed on *E. coli* spheroplasts, and the isolation of high affinity antibodies has been demonstrated (Mazor et al. 2007; Van Blarcom et al. 2010). Also, it has been shown that engineered aglycosylated antibodies expressed in bacteria can mediate a unique effector function that glycosylated counterpart cannot perform (Jung et al. 2010). Ultimately, the APEx display of whole IgGs could be exploited for the isolation of some weak binders (weak as single chain antibodies but stronger as whole IgGs) and may thus facilitate the selection of functional IgGs specific to antigens on the cell surface from large combinatorial libraries.

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