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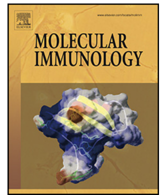

Optimized selection of anti-tumor recombinant antibodies from phage libraries on intact cells

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ABSTRACT

Generation of human recombinant antibody libraries displayed on the surface of the filamentous phage and selection of specific antibodies against desirable targets allows production of fully human antibodies usable for repeated administration in humans. Various lymphoid tissues from immunized donors, such as lymph nodes or peripheral blood lymphocytes from individuals with tumor or lymphocytes infiltrating tumor masses may serve as a source of specific anti-tumor antibody repertoire for generation of tumor-focused phage display libraries. In the case of lack of tumor-associated antigens in the purified form, high affinity anti-tumor antibodies can be isolated through library panning on whole cells expressing these antigens. However, affinity selection against cell surface specific antigens within highly heterogeneous population of molecules is not a very efficient process that often results in the selection of unspecific antibodies or antibodies against intracellular antigens that are generally useless for targeted immunotherapy.

In this work, we developed a new cell-based antibody selection protocol that, by eliminating the contamination of dead cells from the cell suspension, dramatically improves the selection frequency of anti-tumor antibodies recognizing cell surface antigens.

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1. Introduction

Addressed delivery of therapeutics to tumor cells, termed "targeted therapy", is now considered the method of choice in the oncology field. Application of unconjugated monoclonal antibodies, triggering for a specific anti-tumor immune response (McLaughlin et al., 1998; Coiffier et al., 2002; Leonard and Link, 2002), as well as the use of antibodies as drug delivery vehicles of toxins (Lundin et al., 2002; Sievers et al., 1999; Kreitman et al., 2001) or radioactivity (Witzig et al., 2002; Kaminski et al., 2000) represent valid complementations of the traditional cytotoxic therapies (Adams and Weiner, 2005). Tumor therapy with monoclonal antibodies proved to be effective and more tolerable compared to chemotherapy (Kreitman, 2006). In fact, top selling biological are anti-cancer monoclonal antibodies.

One of the efficient methods for developing human monoclonal antibodies is provided by recombinant DNA technology. Generation of large human recombinant antibody libraries, displayed on the surface of filamentous phage, and selection of high affinity binders against desirable targets leads to the engineering and production of fully human antibodies usable for repeated administration in patients. Such antibodies do not elicit harmful immune response as compared to mouse monoclonal antibodies inducing HAMA (human anti-mouse antibody response) thus limiting their application (Maher et al., 1992).

Various lymphoid tissues from immunized donors, such as metastasized or drained lymph nodes from individuals with tumor (Clark et al., 1997; Yip et al., 1997; Graus et al., 1998; Rothe et al., 2004; Xu et al., 2004), lymphocytes infiltrating primary tumors (Wu et al., 2002; Coronella et al., 2002; Kotlan et al., 2005; Pavoni et al., 2007a) as well as the peripheral blood lymphocytes (Hall et al., 1998) may serve as a source of specific anti-tumor antibody repertoire.

Recently in our laboratory we demonstrated the capacity of tumor-infiltrating B lymphocytes to produce, within the tumor microenvironment, antibodies able to recognize cancer cells (Pavoni et al., 2007a). Several scFv recombinant antibody libraries derived from B lymphocytes infiltrating primary breast tumors were generated and panned against known cancer antigens, such as CEA and MUC1, and against living MCF7 breast carcinoma cells. We selected a panel of tumor-specific antibodies from the

Abbreviations: BSA, bovine serum albumin; CEA, carcino embryonic antigen; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; PEG, polyethylene glycol; PFU, plaque-forming unit(s); RT, room temperature; scFv, single-chain variable antibody fragment; SEREX, serological identification of antigens by recombinant expression cloning; TIL, tumor-infiltrating lymphocytes.

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mentioned libraries. The antibodies, that were reactive with purified tumor antigens, were also capable to recognize cancer cells exposing these antigens on their surface. On the other hand, the antibodies obtained by cell-based selection, recognized exclusively intracellular tumor-specific antigens, as it was verified by fluorescent and immunohistochemical staining, and by flow cytometry (Pavoni et al., 2007a). These results are in agreement with the findings of the Hoogenboom's group that previously demonstrated that the local humoral immune response in patients with colorectal carcinomas is biased toward intracellular antigens (Roovers et al., 2001). It is quite common that naturally occurring antibodies recognize preferably intracellular tumor antigens. Also in SEREX-based screening (Sahin et al., 1995) and related methods (Sioud and Hansen, 2001; Minenkova et al., 2003; Pavoni et al., 2004), where tumor cDNA libraries are expressed in bacteria and specific tumor antigens inducing immune response in autologous patients are identified by antibodies from patient sera, the same phenomenon was noted. The antigens identified by SEREX are prevalently cytoplasmic proteins. Cytoplasmic proteins are probably more immunogenic in general, because they are more abundantly expressed (about 90% of cell proteins by mass), and are more easily subjected to proteolysis and MHC presentation than membrane-associated proteins (Coronella-Wood and Hersh, 2003). We also found that antibodies from the TIL-derived library are frequently directed to cytoplasmic antigens being selected when damaged cells and cell debris are present in the affinity selection process.

Taking into account that, an anti-tumor antibody intended for *in vivo* diagnosis or therapy of cancer should specifically recognize the surface of the tumor cells, we aimed to improve the selection efficiency of antibodies recognizing surface tumor antigens. In the present work we describe a new cell-based antibody selection method that dramatically improves selection of surface-specific antibodies by eliminating intracellular targets from the cell preparation used for panning.

2. Methods

2.1. Cell lines

The breast carcinoma cell lines MCF7 (ATCC nr: HTB-22), and MDA-MB-468 (ATCC nr: HTB-132) were maintained in DMEM/F12, supplemented with 10% FBS and used for cell-based panning or for cell-ELISA. MDA-MB-231 (ATCC nr: HTB 26) cells were cultivated in DMEM supplemented with 10% FBS and 1% L-glutamine. Immortal breast epithelial cells MCF10-2A (ATCC nr: CRL-10781) (Soule et al., 1990) were propagated according to the manufacturer's instruction and used as a negative control in FACS analysis.

2.2. Phage libraries

The construction of the two TIL-derived libraries named mixTIL and scFvB96 used in this study was described earlier (Pavoni et al., 2007a). Both of them were generated by using cDNAs derived from breast cancer specimens, characterized by oligoclonality of the immune response. The antibody gene repertoire of the library scFvB96 was generated from tumor specimen of a single patient, while the library mixTIL derives from 7 different samples of breast cancer.

2.3. Phage amplification

Forty μL of scraped bacterial cells were incubated in 40 mL of LB containing 100 μg/mL of ampicillin and 1% glucose until OD = 0.2. The bacteria were collected by centrifuging and resuspended in 40 mL of the fresh LB with ampicillin without glucose. About 6×10^9 PFU (plaque-forming units) of helper M13K07 were added to each mL of the cell suspension, incubated for 15 min at 37 °C without agitation and for another two h in a shaker. Kanamycin was added to obtain a final concentration of 20 μg/mL, and cells were incubated overnight at 32 °C. Phage was purified according to standard PEG/NaCl precipitation (Sambrook et al., 1989).

2.4. Cell-based ELISA

The cells were grown in a 96-well plate until almost confluent. After discarding the growth medium, 100 μL of freshly prepared 4% paraformaldehyde (#15710, Electron Microscopy Science, Hatfield, PA) in PBS were rapidly added to the well and incubated for ten min. The fixing solution was removed by pipetting and cells were incubated with blocking buffer (5% milk, in PBS) for 30 min at RT. PEG-purified phage in blocking buffer (1:1) was added to the cells and incubated for an hour at 37 °C under gentle agitation. The cells were washed three times with washing buffer (0.05% Tween 20 in PBS) and incubated with an anti-M13 HRP-conjugated antibody (27-9421-01, Amersham Biosciences, Piscataway, NJ) for 30 min at 37 °C. The cells were washed five times and the immunoreaction was developed by incubation with TMB liquid substrate (T8665, Sigma) for 15 min at RT and stopped by the addition of 25 μL 2 M H_2SO_4. The results were expressed as the difference between absorbances at 450 and 620 nm, determined by an automated ELISA reader. All assays were done in triplicate.

2.5. Flow cytometry (FACS) analysis

One hundred μL of phage suspension in TE (about 3×10^{10} TU) were preincubated with 50 μL of 4% non-fat dried milk in PBS buffer for 15 min at RT under agitation to block unspecific binding. The phage sample was then added to 5×10^5 human cells in 50 μL of 1% BSA in PBS and incubated for one h at 4 °C in a 96-well plate. After two washings with 1% BSA in PBS, a murine anti-M13 monoclonal antibody, diluted 1/50, was added to the cells and incubated for 30 min at 4 °C. Afterwards, the cells were washed as above and incubated with an anti-mouse PE-conjugated antibody (550589, BD Biosciences Pharmingen), diluted 1/100, for another 30 min at RT. After staining, the cell samples were washed twice. Specific binding of the phage particles displaying scFv antibodies was measured by FACSArray or FACSCalibur instrument (BD Biosciences, Franklin Lakes, NJ). Viability detection was performed by adding 2.5 μL of 7-AAD staining solution (559925, BD Biosciences Pharmingen) to each sample. For FACS analysis with soluble scFv antibodies an anti-FLAG secondary antibody (F3165, Sigma) was used.

2.6. Selection of antibodies from phage-displayed library on intact cells

MCF7 semiconfluent cells (about 10^7) were rinsed three times with PBS and incubated with 2 mL of 2 mM EDTA in PBS for 15 min at 37 °C. Eight mL of PBS containing 10 mM $MgCl_2$ were added to the cells, which were accurately collected by pipetting. The cells were pelleted by centrifuging, washed once with 10 mL of PBS/$MgCl_2$ and finally resuspended in 450 μL of freshly prepared blocking buffer: 3% BSA fraction V (Roshe, #10735086001, Mannheim, Germany), 10 mM MgCl2, 2 mM CaCl2, 5×10^{11} PFU of f1 UV-killed phage in PBS. The use of purified BSA fraction V is important not to compromise the interaction of biotin/streptavidin. Then 30 μl of biotinylated annexin V (Roche, Mannheim, Germany,) were added to the cells and incubated for 5 min at RT on slowly rotating wheel. Then the cells were incubated with 50 μl of Dynabeads (Dynal, Oslo, Norwey) and most of the dead cells were captured by using

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