Panning Phage Antibody Libraries on Cells: Isolation of Human Fab Fragments against Ovarian Carcinoma Using Guided Selection

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INTRODUCTION

The development of monoclonal Abs against tumor-associated Ags has generated considerable interest in the potential use of these reagents for cancer detection and immunotherapy (1, 2). However, clinical assessment of monoclonal Abs has generally yielded disappointing results, largely because of variable induction of a human anti-mouse immune response that enhances the clearance of the murine monoclonal Abs from the circulation and blocks their therapeutic effects (3). Murine monoclonal Abs are also relatively ineffective as cytotoxic agents and in their ability to recruit effector cells or molecules of the complement system. Thus, recent efforts have focused on the construction of human Ab with reduced immunogenicity and improved effector functions using genetic engineering techniques.

ABSTRACT

The display of repertoires of human antibody (Ab) fragments on filamentous phages and selection by binding of the phage to antigen (Ag) have provided a ready means of deriving human Ab against purified Ag. However, it has been more difficult to obtain phage Ab against an individual Ag of a complex mixture, such as cell surface Ag. Using the technique of "guided selection," we generated human Ab against the high-affinity folate-binding protein (FBP), a cell surface Ag that is over-expressed in many human ovarian carcinomas. The guiding Ab template was provided by the light chain of mouse monoclonal Ab Mov19 (K_{D} = 10^{8} M^{-1}) directed against FBP; this was paired with repertoires of human heavy chains displayed on phages, and the hybrid Ab fragments were selected by binding to an ovarian carcinoma cell line (OVCA3). The selected human heavy chains were then paired with repertoires of human light chains. Further panning led to the isolation of a human Fab fragment, C4, with a binding affinity of 0.2 × 10^{8} M^{-1}. This was highly specific for FBP, as demonstrated by ELISA and flow cytometry data and by immunoprecipitation of the relevant molecule from the cell surface of ovarian carcinoma cells. Moreover, C4 targeted the same or a closely related epitope of the Ag, as did the template rodent monoclonal Ab Mov19. These results suggest the usefulness of guided selection as a simple means to derive human Ab against cell surface Ag for which a rodent Ab is available.

The use of very small Ag-binding fragments, such as Fab or scFv fragments, would also be expected to provide reagents with improved tumor penetration and better pharmacokinetic properties (4-6).

Phage display technology has been used to generate human Ab from immune and nonimmune sources. In this approach, diverse repertoires of Ab fragments derived from the rearranged V genes of lymphocytes of human donors (7) or by assembly of human V-gene segments in vitro (8, 9) are cloned for display of Ab fragments on the surface of filamentous bacteriophage by fusion to the minor phage coat protein (pIII) (10). Phage-bearing Abs with specific binding activities are then isolated by binding to immobilized Ag.

Phage selection is very effective with purified Ag but more difficult with complex mixtures of Ags, such as cell surface Ags, which differ widely in type and expression levels. Very few phage Abs have been derived by selection on cells, and to date only hematopoietic (11, 12) and melanoma cells have been used successfully. In the latter case, an immune Ab repertoire was used (13).

One of the more specific tumor markers previously identified in our laboratory (14) is the α isoform of the FBP, a 38- to 40-kDa glycosylphosphatidyl-inositol-anchored molecule (15-18) that binds folic acid with high affinity. Detailed immunohistochemical analysis and evaluation of mRNA expression indicated that FBP is expressed at very low levels on the cell surface of normal epithelial tissues at medium levels on kidney, lung, and breast and at high levels on placental tissues (14, 19-22); overexpression of FBP has also been detected on approximately 90% of ovarian carcinomas (14, 22) in association with progression of the disease (18). We generated two mouse monoclonal Abs, Mov18 and Mov19, directed to nonoverlapping epitopes of FBP (14). Based on the encouraging clinical results obtained with murine anti-FBP monoclonal Abs (23-25), we sought to generate human phage Ab against FBP using the strategy of "guided selection" (26, 27). This technique is based on chain shuffling originally used to increased the affinity of Ab (28). In guided selection, the light or heavy chain of a rodent Ab serves as a template for pairing of human heavy or light chains, respectively, displayed on filamentous phage. Here, we used the light chain of the murine monoclonal Ab Mov19 as a guiding template and an ovarian cell line overexpressing FBP for selection.

MATERIALS AND METHODS

Cell Lines and Abs. The following human tumor cell lines were used: ovarian carcinomas IGROVI (29), a gift from Dr. J. Bénard (Institute Gustave Roussy, Villejuif, France); OVCA3, SKOV3, and SW626, provided by the American Type Culture Collection (ATCC); OVCA432, kindly provided by Dr. R. Knapp (Dana Farber Institute, Boston, MA); mammary carcinomas T47D, MCF7, MDA MB 35, MDA MB 361, MDA MB 231, Hs578T, and SKBR3 (ATCC); lung carcinomas CALU3 (ATCC) and N592 (kindly provided by Dr. J. Minna, Naval Hospital, Bethesda, MD); epidermoid carcinoma A431 (ATCC); and melanoma Mewo (kindly provided by the late Dr. J. Fogh; Memorial Sloan-Kettering Cancer Center, New York, NY). The murine hybridoma Mov19 (14) was used for mRNA extraction.

Monoclonal Abs MOV18 (IgG1) and MOV19 (IgG2a) directed to FBP (14, 30) and monoclonal Ab 9E10 (IgG1) directed to a myc sequence (31) were
affinity purified from mouse ascites or hybridoma supernatants on protein
A-Sepharose C1-4B (Pharmacia/Biotech). MOV19 and MOV18 Fab were
prepared using the ImmunoPure Fab preparation kit (Pierce, Rockford, IL).

Cloning Monoclonal Ab MOV19 Gene. The V genes of mouse mono-
clonal Ab MOV19 were reverse transcribed, amplified, and assembled to
encode scFv fragments using PCR essentially as described (32) but using the
RAPAS kit (Pharmacia/Biotech) according to the manufacturer’s instructions.
The assembled scFv was cloned into the phagemid pHENI (10). Functional
scFv was selected on a monolayer of OVCA3 cells as described below (see “Selection of FBP-binding Clones”). FBP-binding clones were sequenced (see below).
The light chain (Vκ-Ck) of the same monoclonal Ab was also ampli-
fied from the mRNA of the hybridoma MOV19 using the primers MoCK-
FORNot (5′-CCA GCA TTC TGC GGC GCC CTC ATT CCT GAA
GCT CCT GAC-3′) and VKBACKSfi (5′-CAT GAC CAC GCG GCC CAG
CCG ATG GCC GAC ATT GTC ACC CAG TCT CCA-3′) and cloned into pUC19SNmyc (26), and the sequence was checked against that of the
MOV19 scFv. This plasmid was termed pUCMVO19K.

Phage Libraries. Construction of repertoires of human κ light (Vκ-Ck) and
λ light (Vλ-CA) and heavy (VH-CH1) chains has been described (26). The
heavy chain repertoire (VH-CH1) was cloned in the phage vector fdDOG for
display on the surface of phage fused with pll (fdDOG-VH-Cy1); light chain
libraries were cloned in the phagemid vector pHENI (pHENCllib) (26).

Preparation of Phage Displaying Hybrid Mouse-Human Fab Frag-
ments. Escherichia coli TG1 (33) cells bearing the plasmid pUCMVO19K
were grown at 37°C with shaking in 10 ml of 2X TY-AMP-GLU broth (100
μg/ml AMP and 1% GLU) (34). At A600 of 0.5, the culture was infected with
10^{12} pfu of fd phage from the human VH-CH1 library. After 30 min at 37°C,
the culture was centrifuged (2,500 × g, 10 min), and bacterial cells were resus-
pered in 1 ml of 2X TY broth and plated on a 243 × 243-mm dish (Nunc)
containing 2X TYE-AMP-TET (100 μg/ml AMP and 12.5 μg/ml TET) (34).
After 18 h of incubation at 30°C, plates were scraped into 5 ml of 2X TY;
30 μl of the mixture was inoculated in flasks containing 500 ml of 2X TYE-AMP-
TET and incubated 16 h for 30°C with shaking. Phages were precipitated
with polyethylene glycol from the culture supernatant as described (7) and
resuspended in 5 ml of PBS (yield, ~10^{12} phage/ml).

Selection of FBP-binding Clones. The selection was performed on a
monolayer of OVCA3 cells in 100-mm Petri dishes. OVCA3 cells were
seeded and grown as monolayers in 96-well plates. Individual phage clones displaying heavy and
light chains has been described (26) and human VH genes were compared with
the sequencing primers for the RAPAS kit (SI, S3, S4, and S6; Pharmacia).

For ELISA competition, the selected human Fab C4 (final concentration, 5
μg/ml) was mixed with serial dilutions of purified murine MOV19 Fab or
MOV18 Fab in 100 μl of 2% MPBS and incubated for 1 h at room tempera-
ture. C4 binding was detected by rabbit anti-human λ (DAKO) plus peroxi-
dase-conjugated anti-rabbit Ab (Amersham).

Subcloning, Expression, and Purification. To facilitate purification, the
selected human Fab genes were subcloned into the expression vector
pUC119SfiI-NotHismyc (35), which results in the addition of a hexahistidine
tag at the COOH-terminal end of the Fab gene.

RESULTS

Cloning of Murine V Genes. The light chain of mouse mono-
clonal Ab MOV19 served as the “template.” Both heavy and light chain V-genes of Mov19 were initially cloned for display as scFv in
a filamentous phage vector and shown to bind to OVCA3 target cells
bearing the FBP. Table 1 lists the sequences of the polypeptide chains of
each domain.
Selection of Human Heavy Chain. The light-chain V gene was recloned with the mouse Ck gene on a plasmid vector for secretion of the template light chain from the bacterial periplasm.

Bacteria containing the light chain were infected with fd phage encoding a repertoire (size, \(10^7\) clones) of human heavy chains (VH-CH1). Phages produced by these bacteria displayed hybrid Fab fragments (murine Vk-Ck-human VH-CH1); the heavy chains are fused at their COOH-termini to pill, and their light chain partners associate spontaneously in the periplasmic space (10).

The hybrid murine-human Fab phage repertoire was selected by panning on a monolayer of OVCAR3 cells. Phages that bound to the cells were used to infect bacteria containing the light chain to produce phages bearing hybrid Fab fragments. This process was performed three times, with an enrichment in number colony of \(4.7 \times 10^6\) from the first to the third round of panning. Individual clones from the third panning were grown in 96-well microtiter plates, and the phagemid particles were selected on OVCAR3 monolayers as created (size, \(10^7\) clones) was rescued by infection with helper phage, genes (size, \(10^5\) clones for each chain). The resulting repertoire of clones tested by phage-ELISA bound specifically to the cell lines overexpressing FBP (OVCAR3, IGROV1, and SKOV3) and to two other tumor cell lines that do not overexpress FBP (Mewo and A431). Phages from 7 of 44 clones tested bound to each of the cell lines overexpressing FBP but not to the other two cell lines. All seven clones had the same human VH8B sequence (Table 1).

Selection of Human Light Chain. The VH-CH1 gene of the selected human heavy chain was inserted into the phagemid display vector pHEN1 containing repertoires of human k and \(\lambda\) light chain genes (size, \(10^5\) clones for each chain). The resulting repertoire created (size, \(10^6\) clones) was rescued by infection with helper phage, and the phagemid particles were selected on OVCAR3 monolayers as above. The process was performed a total of four times with an enrichment in number colony of \(2.4 \times 10^6\) from the first to the third round of panning. Individual clones from the third panning were grown in 96-well microtiter plates, and the phages bearing the hybrid Fab fragments were screened for binding by ELISA to three different human ovarian carcinoma cell lines overexpressing FBP (OVCAR3, IGROV1, and SKOV3) and to two other tumor cell lines that do not overexpress FBP (Mewo and A431). Phages from 7 of 44 clones tested bound to each of the cell lines overexpressing FBP but not to the other two cell lines. All seven clones had the same human VH88B sequence (Table 1).

V Gene Family and Predicted CDR Loop Structure. The human heavy chain sequence (human VH88B) appeared to derive from germ line segments DP31 and DP77, and the human C4 light chain sequence appeared to derive from germ line segments DPL10 and DPL6 (Table 1), presumably because of PCR crossover (44) in the construction of the V-gene library. The human D1 light chain sequence was derived from the DPL12 germ line only. The human light chains of C4 and D1 both derive from the human VL2 family, and the human heavy chain VH88B derives from the VH3 family (Table 2).
few similarities in loop structure. Ab loops can be classified into different lengths and structural classes or "canonical" loops (45). In the light chains, the CDR1 canonical loop of mouse Mov19 was 1 residue longer (Table 1) and had a different canonical fold (5 instead of 2) from that of the human light chains (Table 2). No differences in lengths or canonical loops (see Table 2) were observed for CDR2. The murine CDR3 was either 1 or 2 residues shorter than its human counterpart. In respect to the heavy chains, the CDR2 of Mov19 has a different canonical fold than the human heavy chain (2 instead of 3), and the murine CDR3 is 2 residues shorter than the human CDR3.

Binding Specificity of Human Fab Fragments. Phages displaying each of the two human Fab fragments, C4 and D1 (according to their light chains), the hybrid Fab fragment (with human heavy and mouse light chain), and the mouse Mov19 scFv fragment were analyzed by FACS on OVCAR3 cells to confirm their binding specificity for cells overexpressing FBP (Fig. 1). Phage expressing the human Ab fragments showed a pattern of reactivity superimposable on that of phage expressing the mouse scFv of Mov19, with phage C4 reactivity slightly higher than that of phage D1 (MFI, 50 versus 35). MFI of hybrid phage was higher than either the human or murine phage phage variant, consistent with previous observations during the selection of phage libraries against soluble Ags (26).

Soluble human Fab fragments were also expressed, harvested from the bacterial periplasm, and shown by FACS to bind specifically to OVCAR3 cells (Fig. 2). Clone C4 showed higher reactivity than D1, and C4 was selected for purification and specificity analysis on a wide panel of tumor cell lines. The C4 human fragment was purified as described in "Materials and Methods" with a yield of 2.5 mg/ml periplasm, and its purity was checked by HPLC and SDS-PAGE. The HPLC profile revealed a major peak with a retention time corresponding to a protein with an apparent molecular mass of 44,700 Da, similar to that of the murine Fab molecule (42,100 Da). A minor peak (~16% of the total) with an apparent molecular mass of 21,000 Da might represent the dissociated chains of the Fab. FACS analysis indicated identical binding specificity of C4 and Mov19, because only the cell lines recognized by Mov19 were also recognized by C4 (Table 3). The differences in MFI of the two Abs likely reflect the use of different detection reagents (see "Materials and Methods").

The fragment was also able to immunolocalize FBP in frozen sections of ovarian cancer surgical specimens by immunoperoxidase staining; specimens of other histotypes were negative for C4 reactivity (Fig. 3).

The molecular specificity of C4 was further analyzed on OVCAR3 cells in competition binding assay with mouse Fabs Mov19 and Mov18. Fab Mov19 efficiently competed with human C4 Fab, whereas Fab Mov18, even at the maximum concentration tested (100 µg/ml) did not (Fig. 4). These results confirm that the Ab selection procedure targeted the same or a closely related epitope as the murine Ab used to guide the selection. However, the low concentration (IC50, 0.25 µg/ml) of murine Fab sufficient to compete with human Fab binding (5 µg/ml) indicated a lower affinity of C4. Indeed, Scatchard analysis of the binding data for both the Mov19 Fab and human C4 Fab on OVCAR3 cells indicated a Kd of 2 X 106 M-1 that was fivefold lower than that for the murine Fab (1 X 106 M-1).

Immunoblot analysis of biotinylated FBP after immunoprecipitation with C4 revealed a single band at approximately 38,000 kDa migrating at the same position as the corresponding band immunoprecipitated with Mov19 (Fig. 5).

![Fig. 1. FACS analysis of phage-binding activity on OVCAR3 cells. Cells were incubated for 1 h at room temperature with medium (A), murine scFv Mov19 (B), hybrid Fab (murine VK-CX-human VH-CH1; C), fully human Fab D1 (D), and fully human Fab C4 (E). Phage binding was detected by incubation with anti-MI3 Ab followed by FITC-conjugated anti-sheep Ab.](image)

![Fig. 2. Binding activity of bacterial periplasmic extracts on OVCAR3 cells. Cells were incubated for 1 h at room temperature with medium (A), murine scFv Mov19 (B), soluble human Fab D1 (C), and soluble human Fab C4 (D). Binding of soluble fragments was detected with 9E10 monoclonal Ab followed by FITC-conjugated anti-mouse Ab.](image)

| Table 3 Binding specificity of human C4 and murine Mov19 on different tumor cell lines* |
|-----------------|-----------------|-----------------|
| Cell type       | MOV19 % positive | C4 % positive |
| Ovarian carcinoma |                  |                |
| OVCAR3          | 90               | 70             |
| IGROV1          | 87               | 74             |
| OVCA432         | 88               | 85             |
| SKOV3           | 14               | 6              |
| SW626           | 7                | 16             |
| Mammary carcinoma |                 |                |
| T47D            | 75               | 59             |
| MCF7            | -                | -              |
| MDA-MB35        | -                | -              |
| MDA-MB361       | -                | -              |
| MDA-MB231       | -                | -              |
| HS578T          | -                | -              |
| SKBR3           | -                | -              |
| Lung carcinoma  |                  |                |
| CALU3           | -                | -              |
| N952            | -                | -              |
| Epidermoid carcinoma |           |                |
| A431            | -                | -              |
| Melanoma        | -                | -              |
| MEWO            | -                | -              |

* Evaluated by FACS analysis.

** Percent positive cells was obtained after subtraction of negative control from Ab treated cells.

† Mean fluorescence intensity of positive cells.
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Fig. 3. Immunohistochemical evaluation of the binding of human C4 Fab (A) and murine MOV19 (B) on cryostat sections from an FBP-positive ovarian carcinoma. All sections were counterstained with hematoxylin. C, negative staining of C4 on an FBP-negative breast carcinoma.

DISCUSSION

In the present study, we used the technique of guided selection to obtain a human Ab directed against a specific epitope of a tumor-associated Ag (FBP) that is overexpressed on ovarian carcinoma cells. This approach has the advantage that it obviates the need for purification of the cell surface Ag (such as the tumor marker or CD Ag on human lymphocytes) provided that a rodent monoclonal Ab is available.

The selected Ab fragment was highly specific for FBP, as demonstrated by ELISA and FACS binding data and by immunoprecipitation of FBP from the cell surface of ovarian carcinoma cells. The Ab was highly specific to FBP and targeted to the same epitope of the Ag as the template rodent Ab (MOV19). The binding affinity of the human Fab fragment (2 × 10⁷ M⁻¹) was approximately fivefold weaker than that of the murine Mov19. However, the observed affinity is adequate for in vitro studies and should be adequate for in vivo applications such as i.p. injection for metastatic localization of ovarian carcinoma (24). Moreover, it should be possible to improve the affinity by mutagenesis in vitro (46) or in bacterial mutator strains (47). Because of their small size, these Fab fragments are expected to have improved pharmacokinetic properties in terms of biodistribution, penetrence of solid tissues, and clearance from the circulation, thus reducing accumulation in normal organs and enhancing target localization (48, 49).

The principle of guided selection, which involves the use of a (mouse) template chain to help fashion the Ag-binding site of a (human) Ab in combination with a complementary (human) chain from a repertoire, might be expected to select human chains that are structurally similar to the corresponding mouse chain and able to make similar contacts to the Ag. Indeed, earlier work with an anti-hapten Ab of known crystallographic structure showed that several of the critical Ag-binding contacts were retained (26). However, we found no clear structural similarities between the original mouse Ab and the selected Ag binding sites. The lack of structural similarities may reflect, in part, the lack of precise homologues in the human repertoire, as noted already (26).

Comparison with the human germ line sequences indicated that our selected anti-FBP Ab contained some mutations in framework regions and that both human C4 light and VH8B heavy chains appeared to derive from a combinations of two different germ line sequences. This presumably occurs by “PCR crossover” between two different germ line sequences during the assembling of the V gene library. Because crossover events are likely to constitute only a minor fraction of the V-gene library, this suggests that the extra combinatorial diversity of the sequences in the hypervariable loops is essential in this case and may also be important in other case of guided selection.

Our data indicate that completely human Ab can be obtained against a tumor target of interest for which clinical studies with murine monoclonal Ab have given promising results (23–25). Because C4 Ab is entirely of human origin, it is expected to be much less
immunogenic and therefore useful for sequential administration as a therapeutic reagent.

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