Human Single-Chain Fv Antibodies to MUC1 Core Peptide Selected from Phage Display Libraries Recognize Unique Epitopes and Predominantly Bind Adenocarcinoma

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ABSTRACT

The tumor-associated antigen MUC1 is overexpressed and underglycosylated in human adenocarcinomas of diverse origins, such as breast, ovary, and colon. We isolated and describe five human single-chain (sc) Fv antibodies specific for the MUC1 variable number of tandem repeats region isolated by in vitro selection from a large naive phage antibody library containing over 6 × 10^9 different scFv antibodies. A synthetic biotinylated 100-mer peptide corresponding to five tandem repeats of the MUC1 peptide core was used for selection.

Two of the antibodies were highly specific for MUC1 as judged by ELISA and flow cytometry. In immunohistochemistry, antibody clone 10A stained MUC1 in the cytoplasm and membrane of adenocarcinoma cells of breast and ovary, whereas in normal epithelium, only cytoplasmic or no staining was observed. With antibody clone 10B, staining was less pronounced and was not always membrane associated in adenocarcinoma. Determination of the fine specificity of 10A and 10B using a novel “indirect epitope fingerprinting” ELISA showed that both antibodies recognize unique epitopes that have not been described for hybridoma-derived anti-mucin antibodies of mouse origin. The selected human antibodies, like many of the murine MUC1 antibodies, recognize epitopes on the protein core of MUC1 that are abundantly present in the underglycosylated form of cell surface mucin on adenocarcinoma. The best human scFv, clone 10A, appears to distinguish normal cells from adenocarcinoma cells, which makes it an attractive candidate for use in antibody-based tumor targeting.

INTRODUCTION

With the development of the hybridoma technique (1), the use of antibodies as “magic bullets” to destroy tumor cells while leaving the tissues surrounding the tumor intact, as visioned by Ehrlich, became the main objective of research in immunotherapeutics. Mouse MAbs to tumor-associated antigens were extensively tested to determine tumor load in vitro or to target tumors in vivo. One of the tumor antigens validated for this application is MUC1. Polymorphic epithelial mucin (MUC1) is a transmembrane molecule present at the apical cell surface of normal secretory epithelial tissues of breast, ovary, colon, lung, pancreas and other tissues and most likely functions as a protective barrier (2–4). Its total molecular weight is variable and higher than M_r 200,000 (5–8). The extracellular portion of the molecule consists mainly of a peptide core containing a VNTR composed of 20 amino acids including heavily glycosylated serines and threonines. MUC1 has been widely accepted both as a serum tumor marker (9, 10) as well as a target for cancer therapy (reviewed in Refs. 11–13). In contrast to normal epithelial cells, MUC1 is abundantly expressed in a nonpolar fashion and in an underglycosylated form, exposing cryptic peptide and carbohydrate epitopes, which “change” the molecule into a tumor-associated antigen in adenocarcinomas (14, 15). In the last decade, a wide range of mouse MAbs were generated against MUC1, mostly binding to epitopes involving the “PDTR” knob in the VNTR (16). As a cancer-associated circulating mucin, MUC1 is used in breast carcinoma patients to monitor therapy and, in the follow up, for early detection of recurrence (reviewed in Ref. 17). Anti-mucin antibodies have also been used to target adenocarcinoma in humans in biodistribution studies (18, 19) or to determine the localization of tumor cells in diagnosis or to monitor residual disease (20–23) and to study the efficiency of therapy (24).

Murine MAbs were shown to have limitations when used for repetitive therapy in humans because of their xenogeneic character. The human anti-mouse antibody response was shown to hamper multicycle therapy when using anti-MUC1 mouse MAbs (25). To overcome the issue of human anti-mouse antibody responses, mouse anti-MUC1 MAbs were humanized via CDR grafting (26–29). These methods involve the inclusion of possibly immunogenic mouse sequences and could therefore still induce anti-mouse or anti-idiotype response. The derivation of completely human antibodies, with an expected minimal immunogenicity when used in humans, using phage display technology has been described (reviewed in Ref. 30). Repertoires of human scFv or Fab fragments are cloned for display on the surface of filamentous phage, and antigen-specific “phage antibodies” are enriched on immobilized antigen (31). From libraries of a few million cloned antibody fragments derived from an IgM pool of naive human B cells, it was possible to isolate human antibodies of average affinity up to 10^7 M^-1 to many different antigens, including tumor-associated antigens such as MUC1 and carcinoembryonic antigen (31, 32). By screening larger libraries, with a few billion antibody fragments, the chances of recovering antibodies with higher affinity for the antigen of interest were improved, and high affinity antibodies (up to 10^9 M^-1) were isolated to self antigens such as carcinoembryonic antigen (33, 34). One of the advantages of this methods is that, once the antibody selected, it may be quickly shaped to fit the format and size or, after further phage-aided affinity modulation, the binding kinetics to optimally suit a particular clinical application (reviewed in Ref. 35).

Here we present the use of phage display to obtain entirely human scFv antibodies to MUC1 derived from a large naive antibody scFv library (33) containing 6 × 10^9 different clones. Three different selection methods were used to retrieve MUC1-specific antibodies. Selected scFv antibodies were shown to recognize specifically unique epitopes within the VNTR region of the MUC1 peptide core, as measured by a new epitope mapping method with 20 overlapping 20-mers of the VNTR. Our detailed characterization suggests that two
of the selected antibodies are particularly suitable as targeting moieties for the immunotherapy of adenocarcinoma.

MATERIALS AND METHODS

Selection of Human scFv Phage Antibodies to MUC1 and Rescue of Phagemid Libraries

A large human scFv library, containing $6 \times 10^6$ scFv antibodies, was obtained from Cambridge Antibody Technology (Melbourne, United Kingdom) and is a subset of a library described previously (33). The monomer MUC1 peptide NH$_2$-PAHGVTASAPDTRPAGSTAP-COOH was synthesized by sequential condensation using Fmoc/tBu amino acids, and the tandem repeat pentamer MUC1 peptide by fragment condensation of the monomer, as described previously (36). Briefly, MUC1 100-mer was prepared by using an improved convergent solid-phase peptide synthesis. Biotinylation was performed on the protected peptide before cleavage from the resin.0.1 mM biotin in dimethyl formamide was coupled to the protected MUC1 100-mer in 20 times molar excess for 2 h at room temperature using disopropyl carbodiimide/1-hydroxy-benzotriazol as condensing agent. Cleavage and deprotection was performed with a TFA:water:phenol:EDT-thioanisole solution (82:5:5:2.5:5) for 2 h at room temperature. The peptide was precipitated in ice-cold ether, washed several times with ether, dried, dissolved in water, lyophilized, and stored at $-20^\circ$C. The purity of the biotylated peptide was assessed by analytical high-performance liquid chromatography using a Waters 600 LC system with UV 486 detector and a semipreparative C$_18$ column (Synchropack RP-P 250 x 7.8 mm; Synchrom, Lafayette, IN).

Selections of phage libraries were modified from Marks et al. (31). Three different selection methods were compared:

1. **Panning on Peptide-coated Immunotubes**: MUC1 100-mer was coated overnight in coating buffer (at 10 $\mu$g/ml, in 0.1 m carbonate buffer, pH 9.6) and blocked, after washing with PBS (0.15 m NaCl, 8 mm Na$_2$HPO$_4$, and 7.8 mm KH$_2$PO$_4$, pH 7.4), with 2% dried skimmed milk in PBS (M-PBS) for 1 h at room temperature. The library, represented by 10$^{13}$ phages, was blocked in M-PBS, added to the immunotube, and left for 2 h at room temperature; the tube was washed 20 times with PBS, and phages, binding to the coated tube, eluted with 1 ml of 100 mM triethylamine, pH 12 (31). Eluted phages were neutralized with 0.5 ml of 1 M Tris-HCl (pH 7.5). Of the eluate, 0.5 ml was used to infect Escherichia coli TGI cells, grown to the logarithmic phase at $A_600$ of 0.5, and the infected cells were plated for amplification as described previously (31). After infection of TGI for 30 min at 37°C, bacteria were grown overnight at 30°C on agar plates (31).

2. **Panning on Peptide-coated Immunotubes**, Followed by Panning on MUC1-expressing Cells. After two rounds of selection as described in "1." a third selection was performed on the T47D breast cancer cell line, known to express tumor-associated glycoforms of MUC1 (37). Briefly, 5 x 10$^7$ T47D cells and 10$^{13}$ phages were preincubated with M-PBS for 10 min; then phages were added to the cells. After 1 h of incubation, cells were washed 10 times with M-PBS + 10% FCS. Specific phage was eluted, and exponentially E. coli TGI cells were infected as described earlier.

3. **Selection on Biotinylated MUC1 (38)**. Phages (10$^{13}$) and 200 $\mu$g of streptavidin-coated paramagnetic beads (Dynal, Oslo, Norway) were preincubated separately for 1 h at room temperature in 2% M-PBS. Phages were incubated for 1 h with decreasing amounts of biotinylated MUC1 100-mer (500, 100, 20, and 4 $\mu$m for rounds 1, 2, 3, and 4, respectively). Streptavidin beads were added, and the mixture was left for 15 min on a rotating wheel. After five washes with M-PBS, five washes with PBS, 0.1% Tween 20, and five washes with PBS, beads were directly added to exponential growing E. coli TGI cells for elution and infection (see "1."). Before the fourth round of selection, streptavidin binders were depleted by preabsorption on a streptavidin-coated (10 $\mu$g/ml) tube.

The diversity of the selected antibodies was checked by means of DNA fingerprinting (39). The scFv gene of different clones was amplified by PCR using primers pUC-reverse (5'-AGCGGATAACAATTTCACACAGG-3') and fd-tet-seq24 (5'-TTTGGTGTCTTTTCCAGACGTTAGT-3') primers and digested with the enzyme BstNI before analysis on an agarose gel (31, 40).

**Induction of Soluble scFv Fragments, Preparation of Periplasmic Fractions, and Purification of Antibodies**

For ELISA screening, individual clones from the second, third, and fourth round of selection were picked from an agar plate and cultured overnight in a microtiter plate 2xTY AG (31). One $\mu$l of each overnight culture was transferred to a microtiter plate containing 100 $\mu$l of 2xTY, 0.1% glucose, 100 $\mu$g/ml ampicillin (2xTYAG; Ref. 31), and grown at 37°C for 2 h. Isopropyl-thiogalactoside was added to each well to a final concentration of 1 mM. After overnight incubation at 30°C, plates were spun (611 x g for 15 min), and supernatant was used directly in ELISA.

Periplasmic fractions were prepared for testing specificity with flow cytometry and immunohistochemistry. These were made as described (41), starting from 50-ml cultures, and harvested 4 h after the start of the isopropylthiogalactoside induction. Briefly, after centrifugation for 20 min at 3400 x g, the pellet was resuspended in 0.530 ml of ice-cold TES (0.2 m Tris- HCl, 0.5 mM EDTA, and 0.5 mM sucrose, pH 8.0) for 10 min, followed by the addition of 0.590 ml of ice-cold TES/H$_2$O (1:3) and a further incubation of 30 min. Supernatants (21,000 x g for 5 min) contained the periplasmic fractions.

For kinetic analysis, induced cultures were prepared as described above. After 4 h of incubation at 30°C, ice-cold PBS was added to the bacterial pellet, and the mixture was incubated overnight at 4°C on a rotator. Supernatant contained the scFvs.

**ELISA**

Specificity of individual scFvs was assessed by ELISA using indirectly coated MUC1. Biotinylated BSA was applied to a microtiter plate at a concentration of 2 $\mu$g/ml in PBS at 37°C for 1 h. After washing three times with PBS/0.1% Tween 20 and 10 $\mu$g/ml streptavidin in PBS, 0.5% gelatin was added to the wells and incubated for 1 h at room temperature. All incubations were followed by three washes with PBS/0.1% Tween 20. For all of the next steps, M-PBS was used instead of incubations. Biotinylated MUC1 (100-mer) was added to each well (0.5 $\mu$g/ml). As negative controls, plates without the addition of biotinylated MUC1 peptide were used. Supernatants of single clones, 1:1 diluted in M-PBS, were added to different wells. Detection of bound scFv fragments was performed by the addition of 100 $\mu$l of 9E10 antibody (hybridoma culture supernatant, 1:1; Refs. 31 and 42), directed to the Myc-tag expressed at the COOH-terminal end of the scFv, in M-PBS for 1 h. Then 100 $\mu$l of peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako, Glostrup, Denmark) in M-PBS were added for 1 h. Cells were stained with 100 $\mu$l of trinitrobenzidine 0.01% in 0.1 M citrate buffer (pH 5.6) and 0.003% H$_2$O$_2$. The peroxidase reaction was stopped with 2.5 N H$_2$SO$_4$, and the A$_450$ was measured in a microtiter plate reader.

**Fine Specificity of MUC1 scFv Antibodies: Indirect Epitope Fingerprinting**

Blocking experiments were performed with a MUC1 60-mer peptide with amino acid sequence NH$_2$-(VTSAPDTRPGSTAPPAGH)-COOH to confirm specificity (43). To define fine specificities, a method derived from the epitope fingerprinting method (44) and which we call "indirect epitope fingerprinting" was performed (Fig. 1). The scFvs were preincubated with each of the 20 overlapping peptides, starting with the peptide NH$_2$-VTSAPDTRPAGSTAPPAGH- COOH, the first amino acid was sequentially removed and added as the last amino acid. scFv antibodies were preincubated with 100 $\mu$l/ml 60-mer or 50 $\mu$l/ml overlapping peptide in M-PBS for 1 h at room temperature. Then incubation mixtures were transferred to an ELISA plate coated with MUC1 100-mer biotin, and ELISA was performed.

**Sequencing and Homology Plots**

Semi-automated DNA sequencing was performed according to the manufacturer of the ALF-Express (Pharmacia, Uppsala, Sweden). Plasmid DNA was prepared from 50 ml E. coli TGI cell cultures. DNA was purified using the Qiagen Plasmid Midikit (Qiagen, Hilden, Germany), and antibody sequences were determined with the AutoRead Sequencing kit (Pharmacia; Ref. 31) using the oligonucleotides fd-tet-seq24 and pUC-reverse primers. Alignment of scFv and 10A and 10B with germ-line VH and VL sequences was done with the Sanger Center Sequence database program (http://www.sanger.ac.uk/DataSearch/gq_search.shtml).
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Immunohistochemistry

Paraffin-embedded normal and tumor tissues were deparaffinized with xylene and ethanol. Endogenous peroxidase was blocked with 0.3% H2O2 in methanol for 30 min. Slides were preincubated with 2% dried skimmed milk, 0.1% BSA in PBS (incubation buffer). After each incubation, step slides were washed three times with PBS. Incubation with the scFv periplasmic fractions (1:1) for 2 h at room temperature and subsequent incubation with the 9E10 antibody (hybridoma supernatant, 1:1 in incubation buffer) for 1 h was followed by incubation with peroxidase-labeled rabbit anti-mouse immunoglobulin for 1 h. Staining was done with diaminobenzidine at 0.5 mg/ml in 0.05 M Tris-HCl (pH 7.6) and with 0.003% H2O2. Tissues were counterstained with hematoxylin.

RESULTS

Selection of Human Anti-MUC1 Antibodies. The primary aim of our study was to select human phage antibodies against the VNTR of MUC1 that is present at the cell surface of cancer cells. We used a naive phage antibody library, described by Vaughan et al. (33), for our selections. To yield a diverse panel of antibodies to the antigen, with a range of affinities and epitopes recognized, we used a variety and a combination of selection methods. Although direct selections on cancer cells displaying MUC1 would be theoretically feasible, the unavoidable selection of irrelevant cell binding antibodies from such a naive library would necessitate extensive depletion and/or subtraction.

Therefore in our approach, we used synthetically made, purified MUC1 in the form of a 100-mer encoding five tandem repeats; such long multiple tandem peptides attain an epitope structure closer to the native conformation of unglycosylated mucin (16, 36). The selection methods included four rounds of biopanning of the library on coated antigen or four rounds of selections using biotinylated antigen or a combination of panning on purified coated antigen (two rounds) and cell panning (third round). In the latter procedure, we aimed to derive those MUC1 antibodies that recognize cell surface-related peptide core epitopes. The selection data are summarized in Table 1.

Panning of the library on immunotubes with coated MUC1 100-mer showed enrichment with regards to phage titer. MUC1-specific monoclonal scFvs were identified with a specific but low ELISA (<3 × background) signal. In the third round, fingerprint analysis showed the presence of three different antibodies, with one clone dominating the population (50% of the positive wells). This clone dominated completely after the fourth round of selection (results not shown). Selection on cells after two rounds of panning gave a slight enrichment; however, no specific antibodies in ELISA nor whole-cell ELISA were detected (data not shown), suggesting that the preselection on coated MUC1 peptide did not enrich for antibodies specific for cell surface MUC1 epitopes.

Because the coating procedure itself may alter the antigen in its conformation, which may in its turn disfavor the isolation of many different antibodies, we alternatively performed selections using bi-
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Antibody Clone

Fig. 2. Specificity analysis of scFvs by ELISA without (•) or with (Δ) the addition of MUC1 60-mer. IG5, monoclonal control antibody binding to MUC1 peptide core.

otinylated MUC1 100-mer, allowing selection in solution and retrieval of MUC1-bound phages using streptavidin-coated magnetic beads. This procedure gave a better enrichment (in part due to selection of streptavidin-specific phages) and resulted in the isolation of four new antibodies (according to DNA fingerprint) besides the clones isolated previously. Four different antibodies were identified in round 3 (2C, 3E, 10A, and 10B), with one clone (scFv 10A) dominating the population in round 4. One new antibody was found in round 4 (1C). Although we preabsorbed streptavidin binders before the fourth round, the percentage of streptavidin binders was much higher in round 4 (43%) than round 3 (27%), explaining the low enrichment levels of the MUC1-specific antibodies.

Fine Specificity of the Human Antibodies by ELISA, BIACore, and Indirect Epitope Fingerprinting. To determine whether the antibodies were specific for MUC1 peptide, we performed an ELISA with the MUC1 100-mer and blocked the specific binding with the MUC1 60-mer. The selected antibodies gave different ELISA results (Fig. 2). Three antibodies (1A, 1C, and 3E) had a low absorbance, whereas two antibodies (10A and 10B) reacted strongly, with signals above A<sub>450</sub> 1.0 (Fig. 2). Binding was inhibited for all antibodies by a MUC1 60-mer peptide.

The specificity was confirmed in BIACore for the two clones with the highest ELISA signals; both scFvs 10A and 10B were shown to specifically bind to MUC1 60-mer directly immobilized onto the surface of a sensor chip (Fig. 3). There was a strong element of avidity visible; indeed, periplasmic fractions that contained mainly scFv dimers as judged by gel filtration were used for the assay. Thus, the estimated off-rate (10<sup>-3</sup>-10<sup>-4</sup> range) is an overestimate of the dissociation rate of the monomeric antibody-antigen interaction. Purified scFv fragments showed a much faster off-rate, in the 10<sup>-1</sup>-10<sup>-2</sup> s<sup>-1</sup> range, on coated MUC1 100-mer.

To define the fine specificity of the antibodies to the VNTR peptide core of MUC1, we developed an indirect epitope fingerprinting assay. The test measures the binding of the scFv to MUC1 100-mer in the presence of an excess of free, overlapping 20-mer peptide. This approach allowed us to use overlapping peptides that were not coupled to BSA. The fine specificity analysis of three scFvs (10A, 10B, and 3E), chosen for their ELISA signal, revealed that each of the antibodies bound to a different sequence of the MUC1 repeat: the immunodominant epitope in mice DTRPA (3E), an epitope where the Ser is slightly involved; PAPG(S) (10A); and a long epitope, T(R)-PAPGSTAPPAH (10B; Fig. 4).

Antibody Sequence. The sequences of the V-genes of the selected antibodies 10A and 10B are shown in Table 2. Both antibodies use different heavy and light chain genes, with clear differences in CDR3 of both heavy and light chains. The V<sub>H</sub> genes of both antibodies belong to the same V<sub>H</sub> family, are derived from the same germ-line segment, DP75, and show a different number and position of mutations from the germ line. The light chains of both clones are of the VA-1 family. scFv 10A consisted of segment DPL-5, with a crossover with DPL-2, and scFv 10B includes segment DPL-11. Other antibodies derived from the same library frequently use the same light chain segments (33).

Binding of Antibodies to Cells and Tissues. We could determine specific binding in FACS for the high-affinity antibodies 10A and 10B. Both, membranous and cytoplasmic MUC1 was measured, and the fluorescence signal could be specifically inhibited with MUC1 60-mer. Antibody 10A bound specifically to the membranous and cytoplasmic MUC1 of T47D cells (Fig. 5). It reacted with the MUC1-transfected 3T3 mouse fibroblast cell line ETA (data not shown) with the same binding pattern as in T47D cells, whereas binding to MCF-7 cells was lower, and no binding to membranous MUC1 in CaCO2 cells (Fig. 6) was observed. This contrasts with the reactivity of...
antibody 10B, which recognizes a larger epitope, but only detects MUC1 in the cytoplasm of cell line T47D (Fig. 5).

Staining patterns (Fig. 7) of various normal and adenocarcinoma tissues with antibodies 10A and 10B are summarized in Table 3. A membranous staining was seen with both antibodies in adenocarcinoma of ovary and breast but not in the tested colon carcinoma and normal tissues. However, the antibodies did not always stain cytoplasmic MUC1 expressed in normal tissues, and the overall staining intensity was lower than in tumor tissues. The staining intensity was less pronounced with scFv 10B as compared with scFv 10A.

DISCUSSION

We report here the isolation of human antibodies to MUC1 by selection from a large human naive scFv library. The specificity of the MUC1 scFv antibodies was confirmed by specificity and competition ELISA, BIACore analysis, immunohistochemistry, and flow cytometry. The MUC1 antigen that is overexpressed and underglycosylated in cancer is thought to be a good target for therapy. Many mouse MAbs have been produced to the VNTR of MUC1 and used unchanged or humanized in biodistribution studies. We are the first to report the selection of human scFv antibodies that bind MUC1 peptide repeat and also recognize MUC1 on cell lines and bind to the protein core of the membrane-anchored underglycosylated MUC1 in cancer but not in normal tissue of the ovary, colon, and breast.

To retrieve antibodies to MUC1 from a large human naive scFv library, we compared three different selection methods. Only when using biotinylated MUC1 100-mer peptide did we succeed in obtaining Anti-MUC1 antibodies that scored positively in immunohistochemistry and flow cytometry. Although we could obtain one apparently specific antibody using MUC1-peptide directly coated on plastic tube, this selection procedure failed to generate the highly specific 10A and 10B clones. It is likely that peptide coated onto plastic may lead to masking or disturbance of the conformation of some antigenic sites or may make some epitopes nonaccessible. The combination of the panning procedure with cell panning could not rescue MUC1-specific clones from this procedure, although it must be indicated that we did not investigate whether the phage particles carrying anti-MUC1 antibodies may be selected at all via cell panning. It could be envisaged that steric hindrance caused by the glycosylation of the mucin protein core could hamper efficient enrichment on cells. In contrast, when using biotinylated MUC1 peptide for selection, the antigen stays in solution during the procedure and may provide conformations more akin those of the native structure of MUC1.

As determined by indirect epitope fingerprinting, one of the three antibodies tested, scFv 3E, recognizes the PDTRPA sequence, which includes PDTR, the sequence most commonly immunogenic in mice (13, 48). On the other hand, two of our phage antibodies tested bind to unique epitopes rarely described within the VNTR of MUC1. The binding sites for the antibodies 10A and 10B map in adjacent regions (13, 48). On the other hand, two of our phage antibodies tested bind to unique epitopes rarely described within the VNTR of MUC1. The binding sites for the antibodies 10A and 10B map in adjacent regions of the PDTR, PAPG(S) and TRPAGSTAPPAH (Fig. 4), respectively. The mouse antibody BCP9 (49) recognizes a closely related epitope (PAPGSTAP; Refs. 44 and 50). The epitope of this MAb contains a putatively glycosylated threonine residue, and most likely as a consequence reacts with the underglycosylated and not with the glycosylated baculovirus MUC1 form (51) nor with MUC1-containing three moles of GalNAc per repeat (52) and binds only intracellularly (45). In contrast with the BCP9 antibody, the 10A epitope only flanks the binding region. Moreover, the serine is involved in binding, but there is no complete abolishment of inhibition, suggesting that this amino acid is involved in binding but is not a critical contact residue. The epitope of scFv 10A seems to be less easily blocked by S- or T-mediated glycosylation, which is reflected in the broader range of cell binding for the 10A antibody (membranous staining in adenocarcinoma) when compared with BCP9 (cytoplasmic staining; Ref. 45) in immunohistochemistry. An important feature of antibodies derived from mice immunized with native MUC1 is the significant involve-

### Table 2: Deduced amino acid sequence of MUC1-specific antibodies

| Antibody | Residue 50 | Residue 58 | FR1  | 10 | 20 | 30 | 40 | 50 | 60 | FR2  | 70 | 80 | 90 | FR3  | 100 | 200 | 300  |
|----------|-----------|-----------|------|---|---|---|---|---|---|---|-----|---|---|---|-----|-----|-----|-------|
| DP5      |           |           |      |   |   |   |   |   |   |     |    |   |   |     |     |     |       |
| 10A-VH   |           |           |      |   |   |   |   |   |   |     |    |   |   |     |     |     |       |
| 10B-VH   |           |           |      |   |   |   |   |   |   |     |    |   |   |     |     |     |       |
| aMUC1-1  |           |           |      |   |   |   |   |   |   |     |    |   |   |     |     |     |       |
| DP7      |           |           |      |   |   |   |   |   |   |     |    |   |   |     |     |     |       |
| 10A-VH   |           |           |      |   |   |   |   |   |   |     |    |   |   |     |     |     |       |
| 10B-VH   |           |           |      |   |   |   |   |   |   |     |    |   |   |     |     |     |       |
| aMUC1-1  |           |           |      |   |   |   |   |   |   |     |    |   |   |     |     |     |       |
| DPL5/2   |           |           |      |   |   |   |   |   |   |     |    |   |   |     |     |     |       |
| 10A-VL   |           |           |      |   |   |   |   |   |   |     |    |   |   |     |     |     |       |
| 10B-VL   |           |           |      |   |   |   |   |   |   |     |    |   |   |     |     |     |       |
| aMUC1-1  |           |           |      |   |   |   |   |   |   |     |    |   |   |     |     |     |       |
| DPL2     |           |           |      |   |   |   |   |   |   |     |    |   |   |     |     |     |       |
| 10A-VL   |           |           |      |   |   |   |   |   |   |     |    |   |   |     |     |     |       |
| 10B-VL   |           |           |      |   |   |   |   |   |   |     |    |   |   |     |     |     |       |
| aMUC1-1  |           |           |      |   |   |   |   |   |   |     |    |   |   |     |     |     |       |
| DPL11    |           |           |      |   |   |   |   |   |   |     |    |   |   |     |     |     |       |
| 10B-VL   |           |           |      |   |   |   |   |   |   |     |    |   |   |     |     |     |       |
| aMUC1-1  |           |           |      |   |   |   |   |   |   |     |    |   |   |     |     |     |       |
| DPL10    |           |           |      |   |   |   |   |   |   |     |    |   |   |     |     |     |       |
| 10B-VL   |           |           |      |   |   |   |   |   |   |     |    |   |   |     |     |     |       |
| aMUC1-1  |           |           |      |   |   |   |   |   |   |     |    |   |   |     |     |     |       |

Notes:

- Small letters, primer encoded mutations; capital letters, amino acid mutation.
- (Residue 58), cross-over from DP75 to DP25 after CDR2 (residue 65).
- Crossing over between K (residue 53) and R (residue 54) in CDR2 for 10A.
- Crossing over between K (residue 53) and R (residue 54) in CDR2 for 10A.
- Crossing over between K (residue 53) and R (residue 54) in CDR2 for 10A.
- Crossing over between K (residue 53) and R (residue 54) in CDR2 for 10A.
Fig. 5. Binding of MAbs (SM3 and 1G5) and scFvs 10A and 10B to T47D cells in flow cytometry. A, membranous staining. B, cytoplasmic staining. Thin line, negative control; thick line, binding of the antibody; broken line, competition for cell binding with MUC1 60-mer.

Fig. 6. Binding of MAb (SM3) to cell lines CaCO2 (A) and MCF7 (B). Thin line, negative control; thick line, binding of the antibody; broken line, binding of the antibody after blocking with MUC1 60-mer.
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A.

B.

C.

D.

Fig. 7. Immunohistochemical staining with human anti-MUC1 scFv 10A. A–C: breast adenocarcinoma; D: normal breast epithelial tissue. Staining with 10A (A, B, and D) or 9E10 (C) is shown. Left panel, ×30; right panel, ×40. Specific membranous and cytoplasmic staining of breast carcinoma (A and B) and weak cytoplasmic staining in normal tissue (D) is seen.

Table 3 Immunohistochemical staining of tissues with MUC1 peptide-specific antibodies

<table>
<thead>
<tr>
<th>Normal</th>
<th>Thyroid</th>
<th>Fetal lung</th>
<th>Pituitary</th>
<th>Breast, patient 1</th>
<th>Breast, patient 2</th>
<th>Colon</th>
</tr>
</thead>
<tbody>
<tr>
<td>10A</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>C</td>
<td>–</td>
</tr>
<tr>
<td>10B</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>C</td>
<td>–</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Adenocarcinoma</th>
<th>Lung</th>
<th>Thyroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast, patient a</td>
<td>m, C</td>
<td>m, C</td>
</tr>
<tr>
<td>Breast, patient b</td>
<td>m, C</td>
<td>m, C</td>
</tr>
<tr>
<td>Breast, patient cc</td>
<td>m, C</td>
<td>–</td>
</tr>
<tr>
<td>Ovarian</td>
<td>m, C</td>
<td>m, C</td>
</tr>
<tr>
<td>Colon</td>
<td>C</td>
<td>–</td>
</tr>
</tbody>
</table>

\* – negative; c, weak cytoplasmic; C, strong cytoplasmic; m, membranous.

ment of arginine in binding (53). This is illustrated by another antibody closely related to the antibody 10A, i.e., antibody C595 [epitope (T)RPAP], where the substitution of the arginine influences binding capacity (54). For human scFv 10A, this important residue is not involved in binding. The human humoral response to the tandem repeat of MUC1 is also found to be related to the PDTR sequence (55), but recently other immunogenic sequences within PAPGSTAP-PAH have been described (56, 57). We found that the epitope of the 10B scFv was very long, containing 14 of the 20 amino acids in the tandem repeat. The antibody probably recognizes a conformational epitope, present in the 100-mer, formed of five VNTR, but not in the

one tandem repeat 20-mer peptide, as demonstrated by the indirect epitope fingerprint ELISA. This epitope could be masked when MUC1 is glycosylated, as in normal epithelial tissues (44). Our results indicate that the multiplicity of antibodies to various epitopes within the MUC1 tandem repeat, as has been found in cancer patients, is also present in the scFv antibody library used, and that the selection procedure described succeeded in simultaneously isolating scFv antibodies with unique and even overlapping epitopes.

BIAcore analysis (Fig. 2) shows that 10A and 10B antibodies bind with high specificity to the MUC1 60-mer coated onto the sensor chip. The off-rates of the selected antibodies on coated 100-mer are relatively fast for antibodies selected from such a large library (in the range of $10^{-1}$ s$^{-1}$ for 10B and $10^{-2}$ s$^{-1}$ for 10A), that has been reported to contain antibodies with subnanomolar affinities, with an average off-rate of $10^{-3}$ s$^{-1}$ (33). This might well be due to the inherent problem of selection of multivalently displayed antibodies (multivalent due to the theoretically possible pentameric display of antibody-gene III product on phage, or due to dimerization of soluble scFv-antibodies with phage-anchored scFvs) on the repetitive antigen MUC1. The antigen requires this repetitive sequence to retain its full tertiary structure (16). It remains to be seen whether selection of antibody libraries with a more truly monomeric nature, such as Fab libraries, would favor enrichment of higher affinity antibodies.

DNA sequencing of antibodies 10A and 10B revealed that both antibodies have the same $\tilde{V}_H$ germ-line sequence (DP75) with the lower affinity antibody 10B almost completely resembling the germ-line gene (four mutations, only two of which in the CDR regions) and with the higher affinity antibody 10A having 12 nonsilent mutations (Table 2). Besides the homology in usage of the heavy chain segment,
antibodies 10A and 10B also share the use of lambda light chain. The sequence similarity is striking, in particular because both antibodies display a very different fine specificity. The same heavy chain FR1 to CDR2 germ-line sequence was found already in an anti-MUC1 antibody, αMUC1-1 (58). In that case, the antibody was selected by affinity chromatography on Sepharose-MUC1 20-mer peptide of a smaller sized naive scFv library (31), but the ELISA signal of the clone was relatively low, and the antibody did not bind to cells. The clone αMUC1-1 is completely germ line but uses a CDR3 different from the clones isolated in this study. αMUC1-1 also shares with clones 10A and 10B the use of a lambda light chain, although the germ-line sequences are different for all three clones: DPL11 for 10B; VL2.1 (DPL10; Ref. 59) for αMUC1-1; and DPL5 with a cross-over with DPL2 in the CDR2 region for 10A. It would be interesting to see, in the future, whether more human antibodies than the three described have the same features and whether there is a relation between the use of the same germ-line family and the special VNTR structure of MUC1.

The selected antibodies would be useful for immunotherapy only if they bound the MUC1 peptide core present on cancer cells. Special focus was set to the staining of breast cells and tissues because glycosylation-sensitive, and therefore tumor-specific, monoclonal antibodies do not stain the higher glycosylated normal breast epithelial membrane (45, 60, 61). Moreover, MUC1 is expressed in 92% of the breast tumor tissues (61), and >80% of all breast cancer metastasis can be imaged by anti-MUC1 antibodies (25, 62). Both 10A and 10B antibodies bind cells in flow cytometry (Figs. 5 and 6). The antibody 10A binds specifically but with a different pattern to the membrane of the MUC1-transfected cell line ETA (data not shown) and the breast cell lines T47D and MCF7 but not to colon cancer cell line CaCo2, likely reflecting, in inverse, the degree of glycosylation of MUC1 in these cell lines (Fig. 6) as described for the tissues they are derived from (45). There is a great similarity in its binding with glycosylation-dependent monoclonal control antibody SM3, which recognizes a different epitope, the PDTRP sequence (48). Antibody 10B stained the cells only intracellularly in flow cytometry (Fig. 5), indicating that this antibody is more sensitive to glycosylation and may recognize the precursor underglycosylated MUC1 molecule (63). In immunohistochemistry, the 10A and 10B scFv stain, as their monoclonal counterparts, the cytoplasmic and membrane-anchored MUC1 adenocarcinoma tissues of different origin, whereas the staining pattern in normal tissues was less intensive or absent and cytoplasmic. This indicates that both antibodies recognize MUC1 in its under- or non-glycosylated form on the surface of cancer cells and confirms that the membrane-anchored MUC1 in cancer cells can expose almost naked core peptide (61).

In conclusion, the human phage antibodies selected in this study recognize epitopes different from the mouse MAbs that have been described before. However, similar to their murine equivalents, they bind the MUC1 peptide, recognize MUC1 on cell lines, and bind to the protein core of the membrane-anchored underglycosylated MUC1 in cancer cells, with minimal or no binding observed in normal tissue of the adult ovary, colon, or breast.

The selected phage antibodies are suitable starting points for developing completely human antitumor cancer therapeutics: (a) the presence of MUC1 in high amounts and in a nonpolarized fashion on the adenocarcinoma cell confers the antibody access to tumor cells and not to normal cells expressing MUC1; (b) during the life of the cell, membranous MUC1 is continuously internalized and recycled (63). Pietersz et al. (64) showed that the antibody CTMO1, reacting with the RPAP, was internalized and delivered cytotoxic drugs much more effectively than an antibody to the APDTR of MUC1. The scFv 10A epitope overlaps with the CTMO1 epitope and therefore could have the same features as carrier for cytotoxic drugs toward the tumor. Besides the use for direct drug delivery, these antibodies might also block MUC1-induced T-cell suppression (65), which could further enhance the antitumoral effect of these agents.

ACKNOWLEDGMENTS

We thank the people of the European Economic Community concerted action project BMH1-CT94-1462, especially Dr. Bruce Acres (Strasbourg, France) and Dr. Marianna Nuti (Rome, Italy) for advice and their offering of materials. We thank Miranda Bouteuser for technical assistance and Edith van der Linden and Dr. Adriaan de Bruine for help with interpretation of the immunohistochemical staining patterns.

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Human Single-Chain Fv Antibodies to MUC1 Core Peptide Selected from Phage Display Libraries Recognize Unique Epitopes and Predominantly Bind Adenocarcinoma

Paula Henderikx, Maria Kandilogiannaki, Claudia Petrarca, et al.


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