Research article

Generation of antibody-based therapeutics targeting the idiotype of B-cell malignancies

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ABSTRACT

Background: A feature of many B-cell tumors is a surface-expressed immunoglobulin (sIg). The complementarity-determining regions (CDRs) of the sIg, termed the ‘idiotype’, are unique to each tumor. We report on a phage selection strategy to generate anti-idiotype therapeutics that reacts with sIg CDR3 sequences; the MEC1 B-cell tumor line was used as proof of concept.

Methods: To create a mimetic of the MEC1 idiotype, CDR3 sequences from heavy and light chains of the sIg were grafted into a single chain variable fragment (scFv) framework scaffold. Using the Tomlinson I phage library of human scFvs, we enriched for binders to MEC1 CDR3 sequences over unrelated CDR3 sequences.

Results: By ELISA we identified 10 binder phages. Of these, five were sequenced, found to be unique and characterized further. By flow cytometry each of the five phages bound to MEC1 cells, albeit with different patterns of reactivity. To establish specificity of binding and utility, the scFv sequences from two of these binders (phages 1 and 7) were converted into antibody-toxin fusion proteins (immunotoxins) and also cloned into a human IgG1 expression vector. Binders 1 and 7 immunotoxins exhibited specific killing of MEC1 cells with little toxicity for non-target B-cell lines. The full-length antibody recreated from the binder-1 scFv also exhibited specific binding.

Conclusion: Our results establish the utility of using engrafted CDR3 sequences for selecting phage that recognize the idiotype of B-cell tumors.

Statement of Significance: Selecting anti-idiotype antibodies from phage display libraries may overcome the need to immunize either animals or human patients with tumor-related material. In addition, engineering-suitable idiotype mimetics, as described below, will increase the chance of that success.

KEYWORDS: idiotype; lymphoma; immunotoxin; immunotherapy; phage display

INTRODUCTION

Tumor-specific antigens have long been sought as targets for antibody-based therapies [1–5]. One ideal tumor antigen, often called ‘idiotype’, is the unique surface immunoglobulin (sIg) expressed on many B-cell malignancies, including follicular lymphoma, mantle cell lymphoma, chronic lymphocytic leukemia and some diffuse large B-cell lymphomas [6–8]. This surface glycoprotein is expressed uniquely on the patient tumor cells but not on normal B-cells or stem cells. Further, because of the essential nature of the B-cell signaling pathway, this target is likely to be retained to support tumor growth and survival [9]. The portions of the sIg that make it unique reside in the complementarity-determining regions (CDRs) sequences of the variable portions of the heavy and light chains. Further, it has been argued that because of V,D,J/V,D recombination, the CDR3s constitute the...
essential ‘uniqueness’ of the antibody [10]. However, while a unique sequence (the ‘idiotype’) can be defined precisely, generating reagents that react with specific CDRs is a challenging enterprise. Also, because each B-cell tumor expresses a distinct sequence, the production of patient-specific therapeutics becomes logistically quite challenging.

The development of anti-idiotype therapies was pioneered by Levy et al. [6, 11, 12]. Early clinical trials reported on at least one responding patient who received a monoclonal antibody directed to the idiotype of his/her tumor cells [11]. Initially, anti-idiotype antibodies were produced in mice following immunizations with patient cell material. However, the reliance on an animal immunization protocol for the production of patient-specific antibodies was difficult to justify outside of a research setting. Ultimately this led to the development of a different approach whereby patient tumor cells were engineered to generate a hybridoma, producing soluble antibodies corresponding to an individual’s sIg [13–15]. These antibodies were purified, conjugated with Keyhole limpet hemocyanin (KLH) and formulated as a vaccine preparation. Patients received immunoglobulin-KLH to their own idiotype in the presence Granulocyte Macrophage Stimulating Factor (GM-CSF), but only after completing a course of chemotherapy to reduce their tumor burden. Two very similar approaches were tested in the clinic and results that describe the outcomes of several hundred patients [14, 15] were reported. Two features were noteworthy: (1) responses to IgM sIg produced an increase in progression-free survival when compared to patients with an IgG sIg and (2) patients who responded to the vaccine had better outcomes than those who did not. A central feature of this vaccine approach was the reliance on the patient to generate an anti-tumor response to his/her own idiotype. At the time of manuscript submission, despite some encouraging results, this vaccine approach has not been approved for human use. However, as mentioned above, the initial concept devised by Levy et al. was the production of anti-idiotype antibodies in mice followed by an infusion of purified antibodies to target patient tumor cells. While that approach is not easily scaled for the general population, the concept of passive administration of anti-idiotype antibodies remains attractive. Therefore, we sought other means of generating anti-idiotype antibodies that could be administered passively.

Two technologies have come together to allow for the identification and engineering of antibody sequences. First, gene sequencing has matured to the point where, with appropriate primers, it is now possible to amplify and sequence most variable portions of relevant antibody genes [16]. Second, antibody engineering has pointed the way for grafting antibody CDR sequences into the framework scaffolds of well-folding antibodies [17]. We wished to combine both technologies: first to identify unique CDR sequences (actually CDR3 sequences) from the variable portion of a model B-cell tumor and then to use those sequences to recreate the essential uniqueness of that idiotype and thereby generate a ‘target’ for the selection of anti-idiotype antibodies. Finally, it is well understood that not all B-cell lymphomas express an immunoglobulin molecule on their cell surface. For our approach to be valid we chose a target cell that expressed a surface immunoglobulin. As discussed below, we chose MEC1 cells derived from a Chronic Lymphocytic Leukemia (CLL) patient with strong expression of sIg [18].

**MATERIALS AND METHODS**

**Cell culture**

MEC1 cells were purchased from the Leibniz Institute DSMZ German Collection of Microorganisms and Cell Cultures. JVM13 cells were purchased from American Type Culture Collection (ATCC). Nalm6 cells were obtained from Alan Wayne, National Cancer Institute (NCI). Cells were grown in RPMI-1640, 10% fetal bovine serum.

**Preparation of cDNA from MEC1 cells**

Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen, Germantown, MD). Using 5 μg of isolated total RNA, reverse transcription was performed using SuperScriptTM III Reverse Transcriptase kit (Invitrogen, Carlsbad CA). This yielded single-stranded cDNA to be used as a template in IGH and IGL cloning. Primers purchased from BIOMED-2 in combination with AmpliTaq Gold master mix were used in the Polymerase Chain reaction (PCR) reactions. While the primers from BIOMED-2 were able to clone the majority of the variable region for both heavy and light chain, some sequences were incomplete. Therefore, additional primers were designed so that the full heavy and light chain variable regions of MEC1 cells could be sequenced (16). Primer sequences are supplied in Table 1.

**Cloning of variable IGH and IGL from MEC1 cells**

Primers for IGH cloning are listed in Table 1. Both forward and reverse primers were mixed with 8 μl cDNA and 25 μl AmpliTaq Master Mix. Primer mix used in IG kappa chain cloning is also listed in Table 1; primers were mixed with 5 μl cDNA and 0.5 μl pure AmpliTaq Master Mix. Sequence data were queried against the IMGT database to determine V-gene families. The IGH was from V4-59 while the IGK was from V4–1.

**Construction of the 4D5-WT (scaffold) and 4D5-MEC (target) vectors**

The base vector for expression of proteins was a derivative of a pCAC19-pCAC7-based plasmid (19). The key features of the plasmid were a pBR322 origin of replication, chloramphenicol acetyl transferase gene (CAT) for selection and a T7 RNA polymerase promoter driving gene expression. 4D5-wild-type (WT) and 4D5-MEC were generated by DNA synthesis (Blue Heron Biotech, Bothell WA) and include an OmpA periplasm directing signal sequence (cleaved upon entry in the periplasm), followed by a FLAG tag epitope (DYKDDDDK) to allow binding to an anti-FLAG affinity purification resin (Genscript catalog #L00432), the appropriate single chain...
Table 1. Primers used for amplification of the variable portions of the heavy and light chains of the MEC1 sIg

|                         | IGK (IGKV–IGKJ) gene rearrangement |        |
|-------------------------|-------------------------------------|--------|
|                         | Forward                             | Reverse|
| Vk1f/6                  | tcaaggtcacgcrcaatggtcgtcgc         | Jk1–4  |
| Vk2f                    | gcctcttcctctgcaatggctgctgta        | Jk5    |
| Vk3f                    | cccaggtctctcaatcatagcatacc         |        |
| Vk4                     | caactgtaagaagccgacaggttgg          |        |
| Vk5                     | ccctgaagccgacaggaatag              |        |
| Vk7                     | gaccgtttacaccctcactaat              |        |
|                         | IGHV (IGHV–IGHD–IGHJ) gene rearrangement |        |
|                         | Forward                             | Reverse|
| VH1–FR1                 | gcctcaggtgaaggtctctggaag            | JH     |
| VH2–FR1                 | gctgggcctcagctctgtaagacccc         |        |
| VH3–FR1                 | cttggggcctctgcttaccctg             |        |
| VH4–FR1                 | cttcgagacccgctcctacgctg            |        |
| VH5–FR1                 | cgggctctctgctgctggtagg             |        |
| VH6–FR1                 | cttcgagacccgctcctacgctg            |        |

Figure 1. Control (A) and target (B) scFvs for differential selection of phage. (A) Control 4D5 scFv with WT CDR3 sequences. (B) Target 4D5 scFv with grafted tumor CDR3 sequences into both the heavy and light chain variable segments. Each CDR segment of the control scFv is displayed in yellow while the CDR3s of the target are in red.

variable fragment (scFv) sequence and a termination codon. Restriction sites flanking this Open Reading Frame (ORF) were NdeI and EcoRI for cloning purposes. Amino acid sequences of both constructs are represented as a cartoon (see Fig. 1) and in detail in Supplemental Fig. 1.

Expression of 4D5-WT and 4D5-MEC

*Escherichia coli* BL21star (DE3) (Invitrogen) were transformed with either the p4D5-WT or p4D5-MEC1 plasmid DNA. Cultures for expression (LB broth + chloramphenicol [25 μg/ml]) were inoculated with the transformations and grown at 30°C with shaking at 250 rpm for 48 h using the Overnight Express™ autoinduction system (Novagen, Madison WI). We exploited a leaky T7 promoter to give slower expression allowing more soluble protein production. Cultures were centrifuged gently (1800g for 20 min) and pellets were resuspended in 15 ml of 40% sucrose lysis buffer (40% sucrose, 20 mm Tris, pH 7.5 and 1 mm EDTA) + protease inhibitors (ROCHE Complete EDTA free cocktail #11873580001) for 30 min. To remove insoluble material, samples were centrifuged at 15 000 rpm for 45 min and the supernatant retained. The soluble fraction was mixed with three volumes of dilution buffer (225 mm NaCl, 50 mm Tris, pH 7.5 and 1 mm EDTA) to lower viscosity and increase the salt concentration. To remove aggregates material was passed through a 0.22 um filter. Following the manufacturers protocol the filtered sample was applied to anti-FLAG resin at 10°C, washed and dislodged with an acidic elution buffer (0.1 M glycine-HCl at pH 3.5). The eluate was neutralized using 100 mm Tris, pH 8.0. Samples underwent a final purification on a Superose 6 gel filtration column (GE Healthcare, Chicago IL) with Phosphate buffered saline (PBS) as the mobile phase.

Enrichment for phage binders to 4D5-MEC

The Tomlinson Phage I library (3.45 × 10¹¹/ml) was incubated with a 10% milk-PBS blocking solution for 1 h at room temperature. Then 500 μl of blocked phage were added to purified 4D5-WT protein (200 μg/ml) mixed and allow to interact overnight at 4°C, to remove scaffold binders. To retrieve specific MEC binders, wells of an ELISA plate were coated overnight at 4°C with 10 μg/ml of 4D5-MEC. Wells were then washed twice with PBS-Tween (0.01%) and blocked for a further ~4 h with 10% milk-PBS solution. Preabsorbed phage (50 μl) was added to blocked wells and the plate was incubated at room temperature for 30 min. This was followed by 6 washes of 15 min each. Bound phage was eluted with a glycine buffer at pH 2.0 and then neutralized with the addition of 1M Tris pH 8.0. Recovered phage was then re-titered. The panning step was repeated. After a third round, eluted phage was isolated from single colonies.
Phage ELISA

Individual colonies were grown to produce clonal phage. Approximately 200 individual phage preparations were incubated coordinately on plates that had been coated with either 4D5-WT or 4D5-MEC. Bound phage was detected with an anti-M13 monoclonal antibody Horseradish Peroxidase (HRP) conjugate (GE Healthcare Life Sciences #27942101). Those wells where the absorbance in the 4D5-MEC wells exceeded the 4D5-WT by more than 2-fold were retained. Of 10 candidate phage, 5 were brought forward for sequencing of the encoded scFv. See Supplemental Table 1 for absorbance values of phages 1 to 10.

Phage scFv sequencing

To characterize individual binder phage, each scFv insert was sequenced. Sequencing primers are listed below while sequence data are discussed in the results section and presented in detail in Fig. 3 (amino acid sequence) and Supplemental Fig. 3 (DNA sequence).

MEC1 flow cytometry: characterization of phage

Individual binder phage was evaluated for reactivity with intact MEC1 cells. Phage at (1 × 10^9/ml) was added to MEC1 cells and binding detected with a mouse monoclonal antibody to M13 phage coat protein (ThermoFisher MA1-12900).

Generation of immunotoxins

To ensure that binding to MEC1 cells was mediated by the scFv portion of the phage, we generated soluble scFv proteins including immunotoxins. This allowed for the measurement of binding directly by flow cytometry and for cell killing, which was used as a surrogate for binding and internalization.

MEC binder phage (1 and 7) were used as template DNA for PCR. Oligonucleotides were designed to add restriction sites for cloning.

**MEC NdeI FOR:** 5’CATATGGCAGGTTGACCT-GTTGG 3’

**MEC HindIII REV:** 5’ AAGCTTTGATTTCCAC-CATTGCC 3’

PCR reactions are as follows: 95 degrees/5 min; (95 degrees/1 min., 60 degrees/1 min., 72 degrees/1 min) 29 cycles, 72 degrees 10 min final cycle.

The resulting PCR products cloned into T7 expression vector pRB 504 (SS1P) via NdeI-HindIII to yield pMEC(1 and 7)-PE38.

Phage scFv sequencing primers are as follows:

**pIT2 FOR:** 5’ CATATGGCCAGGTTGACCT-GCTG 3’

**pIT2 REV:** 5’ AAGCTTTGATTTCCAC-CATTGCC 3’

Expression and purification of phage derived scFv-PE38 immunotoxins

_E. coli_ BL21star (DE3) was transformed with either binder-1 or binder-7-PE38 plasmid DNA. Cultures for expression (LB broth + chloramphenicol [25 μg/ml] + overnight express system1 (Millipore cat 71300-4)) were inoculated with transformants and grown at 37°C in a 2 L baffled flask with shaking at 250 rpm for 20 h. Protein expression via the T7 promoter was induced automatically with the overnight express additives. Cultures were centrifuged (4 000g for 10 min) and pellets resuspended in 10 ml of 50/20 TE (50 mM Tris, 8.0, 20 mM EDTA) and cells were lysed with lysozyme (Sigma cat L-7651 75 mg/1 L culture) for 1 h at room temperature. The pellet was collected via centrifugation at 18 000g. The pellet containing insoluble immunotoxin was resuspended in 22.5 ml of 50/20 TE + 2.5 ml of 25% Triton x-100. The pellet is further washed four times with Triton x-100 (1% final) and four times without Triton x-100. The final pellet was resuspended in 10 ml solubilization buffer (6M Guanidine-HCl, 0.1 M Tris-HCl, pH 8.0, 2 mM EDTA and 65 mM dithioerythrol) and rocked at room temperature for 17 h. The solubilized inclusion bodies (5 ml at ≤5 mg/ml) were added to a slowly stirring 1 L of refolding buffer (0.1M Tris pH 8.0, 0.5 M L-Arginine-HCl, 2 mM EDTA, 8.0, 0.9 mM GSSG (gluthionate, oxidized) and pH 8.0). The preparation was dialyzed against 20 mM Tris-HCl, pH 8.0 and 100 mM Urea and then applied to Q-Sepharose resin (GE Healthcare cat 17-0510-01) and eluted with a 0–60% NaCl gradient elution (using 20 mM Tris–HCl, pH 8.0 + NaCl). Peak fractions were pooled and concentrated using an Amicon Ultra concentrator (3 000 MWCO EMD Biosciences cat UFC903024). Lastly, the concentrated sample was applied to a Superose 6 gel filtration column (GE Healthcare).

Cell viability assays

Binders 1 and 7-scFv immunotoxins were assayed against MEC1 and non-target cells (JVM13 and Nalm6) to measure reductions in viability. To quantify viability at 72 h post-immunotoxin addition, CellTiterGlo (Promega, Madison WI) was used to determine cellular Adenosine Triphosphate (ATP) levels.

Cloning into pFUSEss-CHIg-hG1 and pFUSE2ss-CLIg-hK vectors

Binders 1 and 7 scFvs were cloned into human IgG1 expression vectors (Invivogen, San Diego CA). Antibodies were expressed from 293 T cells via transfection with the appropriate plasmids in the presence of lipofectamine. Plasmid DNA was prepared using Qiagen-Midi kit and plasmid DNA was transected in the ratio of 1:2. Secreted antibody was collected at 48 and 72 h post-transfection and subjected to protein A purification. A two-stage elution protocol was used to first elute bovine IgG (at pH 5.0) and then human IgG1 (at pH 2.0).
Flow cytometry: characterization of immunotoxin and antibody binding

To characterize immunotoxin binding, cells were incubated with purified proteins at 5 μg/ml. The presence of cell-bound PE38 was detected with a mouse monoclonal antibody to pseudomonas exotoxin, M40-1 (5 μg/ml) [20] and revealed with a goat anti-mouse phycoerythrin (PE). Controls included cells alone and cells plus secondary antibodies but no immunotoxin (see Fig. 5A).

To characterize full length antibody binding, cells were incubated with either binder-1 or binder-7 at 10 μg/ml. As an isotype control for non-specific binding, MORAb-009 [21] (gift of Dr. Raffit Hassan, NCI) was added to cells also at 10 μg/ml. All experiments included an Fc receptor blocking step (Biolegend, human TruStain FcX, San Diego CA). Antibody binding was detected with a PE-labeled anti-human donkey F(ab’)2 (Jackson ImmunoResearch Labs, West Grove PA).

RESULTS

Vector design for engrafting CDR3 sequences into a scFv scaffold

To select idiotype-reactive phage, we first designed a vector that could accommodate the CDR3 sequences derived from the heavy and light chains of the sIg of a B-cell tumor. We chose a stable ‘scaffold’ vector based on the scFv from the 4D5 antibody [17]. The cDNA encoding the 4D5 scFv was synthesized, preceded by an OmpA signal sequence and a FLAG tag—see Fig. 1A. The CDR3 sequences of the 4D5 antibody were retained and served as a negative control for the panning step. To produce a target vector for positive selection of phage, the CDR3s of 4D5 were replaced with the heavy and light chain CDR3s of the tumor cell (Fig. 1B). From these two vectors, scFv proteins can be expressed to the periplasm of E. coli, purified via an anti-FLAG affinity column and gel filtration chromatography and made available for negative absorption and positive selection of phage.

Sequencing of variable domains of MEC1 immunoglobulin gene

To generate antibodies to the idiotype of the MEC1 B-cell tumor line, we first determined the CDR3 sequences of heavy and light chains of the unique rearranged immunoglobulin gene (see Fig. 2A and B and below). MEC1 immunoglobulin cDNA was prepared and sequenced with primers as listed in Table 1. Clones were sequenced and the variable portions of the heavy (IgM/D) and light chain (kappa) were determined. A similar partial sequence was reported previously [18]. Also, as reported, the heavy chain DNA sequence was from the IGHV 4–59 gene. The locations of CDR1, 2 and 3 sequences were determined via queries of each variable chain to the IMGT database (http://www.imgt.org/) and are listed as color-coded amino acids in Fig. 2.

With CDR3 sequences in hand, we grafted these into the scFv vector. A sequence comparison of CDR3 sequences from the 4D5 WT and 4D5 with CDR3 grafted from MEC1 revealed 8 differences from the 13 amino acids in the heavy chain CDR3 and 4 differences from the 9 amino acids in the light chain CDR3 (Fig. 2C). (A full sequence comparison of the variable regions of 4D5 and MEC1 is provided as Supplemental Fig. 2). After producing purified proteins corresponding to each vector, phage panning was used to select for specific binders to the scFv containing the MEC1 CDR3s.

Selection and identification of binder phage

The Tomlinson I phage library contains approximately $1.4 \times 10^8$ distinct clones derived from a non-immune human source [22]. Phage was first negatively selected with the 4D5-WT protein and then positively adsorbed to the target 4D5-MEC protein. Three rounds of enrichment were performed. To isolate individual M13 phage that bound 4D5-MEC, single colonies were picked and phages were grown from these single colonies and then evaluated by ELISA, where potential binders were identified as those that scored with a greater than 2-fold preference for 4D5-MEC over 4D5-WT. Ten such phages were identified from approximately 200 candidates.

Characterization of binder phage

From 10 candidate binder phage, 5 were selected initially for further characterization. First the sequence of each scFv insert was determined. Sequencing confirmed that each of the five isolates was unique, in both DNA (Supplemental Fig. 3) and amino acid sequence (Fig. 3). Of note, two of the five phages (binders 5 and 10) contained Amber stop codons in-frame with the protein coding sequence. Because the TG-1 vector used to propagate the phage harbors an Amber suppressor, these would be expressed as full-length proteins in this strain. To compare isolates, the amino acid sequences of all five binders were aligned (Fig. 3).
Figure 3. Alignment of amino acid sequences from each of the scFVs derived from five binder phages. Amino acid sequences designating CDR1, CDR2 and CDR3 are shown in green, blue and red, respectively. In the case of binders 5 and 10, ‘tag’ stop codons are shown as a red ‘X’. At position 180, L to H was noted for phages 5 and 7 and is colored light blue. The order is heavy chain, a glycine-serine linker and then the light chain. Where side chains are mutated, within the parameters of the Tomlinson library construction, amino acids are highlighted in yellow. The corresponding DNA sequence is provided in Supplemental Fig. 3.

alignment we confirmed that each phage encodes a unique scFv. We note also that the TAG codon in binders 5 and 10 occurred in the framework regions of the scFv structure. These amino acids would be changed to glutamine and tryptophan, respectively, to restore the framework regions to a WT sequence. Comparing the sequences of the five phages, differences were noted at CDR2 and CDR3 locations of the heavy and light chains (Fig. 3) reflecting the diverse side chains residues that were engineered into the original Tomlinson I Library.

Reactivity of phage with MEC1 cells
To evaluate reactivity for cells, phage isolates, 1, 5, 7, 9 and 10 were incubated with MEC1 cells and then probed with an antibody to phage pIII (Fig. 4). Phages 1, 7 and 9 showed complete reactivity with all cells shifting to the right compared to control incubations with antibody reagents but no added phage. Phages 5 and 10 appeared to react with only 50% of the cells. The basis for this was not investigated further. However, based on the relatively strong reactivity by phages 1 and 7 the corresponding scFvs were cloned from the phage vectors and inserted into toxin expression vectors such that binders 1 and 7-toxin proteins were generated which could be evaluated in both cell binding and killing assays. The scFvs were fused to domains II and III of Pseudomonas exotoxin (a truncated form of the toxin called PE38) to form so-called immunotoxins [23]. Phage 9 was not investigated further.

Immunotoxin action of MEC1 binders
Binders 1 and 7 immunotoxins were expressed in E. coli and purified by standard methods [24]. Binding to MEC1 cells was initially established via flow cytometry. Binder-1 or binder-7 immunotoxins were incubated with MEC1 cells and binding confirmed with the addition of an anti-toxin monoclonal antibody Fig. 5A. While both immunotoxins exhibited specific cell binding, binder-1-PE38 exhibited stronger interaction with MEC1 cells (Fig. 5A). To assess the specificity of binding leading to cell killing, binder-1 or binder-7 immunotoxins were added to either target MEC1 cells in a 3-log range (5, 50, 500 and 5 000 ng/ml) or were added at the same concentrations to non-target JVM13 or Nalm-6 cells (Fig. 5B, C and D). Data indicated that MEC1 cells were more sensitive to the immunotoxins than JVM13 or Nalm-6 cells. There was no evidence that the non-target cells were toxin resistant as each of the three B-cell lines were killed by the immunotoxin, HA22, directed to CD22 (Fig. 5B, C and D). From these data we conclude that it is possible to generate soluble therapeutic scFv-based proteins from the corresponding binder phage that retain reactivity for the idiotype of the target sIg displayed on living cells.

Production of full-length antibodies
In the cancer setting, most currently approved antibody-based therapeutics are full-sized human, humanized or
Figure 4. Flow cytometry of phage binding to intact MEC1 cells. Cells were incubated with candidate phage and binding detected with antibodies to pIII of the phage coat and a PE-labeled secondary antibody. A control incubation, with the addition of primary and secondary antibodies, was also conducted. The gating highlights the segment designated as ‘P3’ for control binding and ‘P4’ for specific binding of phage to MEC1 cells.

Figure 5. Binding and cytotoxicity of immunotoxins for B-cell tumor lines. The scFvs of binders 1 and 7 phages were cloned into a toxin vector (PE38) to make single chain immunotoxins. (A) Binding to MEC1 cells was confirmed by flow cytometry using antibody to the toxin. Unstained and anti-toxin controls are shown in red and orange. Binders 1 (left) and 7 (right) histograms are shown in light blue. Binders 1-scFv-PE38 and 7-scFv-PE38 (5, 50, 500 and 5 000 ng/ml) were incubated with (B) MEC1, (C) JVM13 or (D) Nalm6 cells. The immunotoxin HA22, directed to CD22 was used as a positive control. Viability was determined after 72 h using the CellTiter-Glo® assay to measure ATP levels.

human–mouse chimeric antibodies. To produce human antibodies from our scFvs, we cloned the scFvs of binders 1 and 7 into the pFUSE vectors encoding human IgG1 heavy and kappa light chains. Transfection of 293 T cells was used to produce candidate antibodies. From supernatants we isolated binders 1 and 7 antibodies and evaluated their binding against target MEC1 cells and non-target JVM13 cells. The monoclonal antibody, MORAb9, which binds surface mesothelin on mesothelial cells and some cancers, was used as an isotype control [21]. By flow cytometry, binder 1 clearly bound target cells but failed to bind the non-target cell (Fig. 6). Binder 7, however, did not
Figure 6. Binding of ‘binder 1’ monoclonal antibody to cells. (A) Binding to MEC1 cells and (B) binding to JVM13 cells. MORAb-009 antibody, also an IgG1 (reactive with surface mesothelin on epithelial tumors), was used as an isotype control. Red, no primary antibody; blue, isotype control; green, binder 1 antibody.

demonstrate binding activity above the isotype control. The basis for lack of binding by binder 7 antibody was not investigated further. Based on results with two scFv-based immunotoxins and the full-length antibody ‘binder 1’, we conclude that it is possible to generate antibody-based molecules that can react with the idiotype of a B-cell tumor. Further, there is experimental support for the approach of using CDR3-targeting to select phage that are reactive for B-cell tumors.

DISCUSSION

The original goal for anti-idiotype therapy, as described by Levy et al., was the passive administration of an antibody or antibodies targeting the CDRs of the patient’s tumor cells [11]. In the ‘bygone’ era of therapeutic mouse monoclonal antibodies, this entailed the injection of patient-derived immunoglobulins into Balb/c mice followed by screening and the selection of the appropriate mouse anti-idiotype hybridomas. Monoclonal antibodies were prepared from these hybridomas and injected into patients as therapeutic agents. Clinical results indicated that objective responses could be achieved, although most were short-lived [25]. Also, ~50% of patients made antibodies to the mouse monoclonal antibody. Therapeutic challenges of this kind led to an alternative approach of generating idiotype vaccines, focused on the re-infusion of tumor-derived antibody linked with a protein adjuvant (KLH) and administered with GM-CSF [14, 15]. This strategy has proven safe and generated objective responses for a limited number of patients. However, overall clinical objectives were not met and the general use of this approach has not been approved [14]. Therefore, other avenues would seem worth exploring.

The particular goal of anti-idiotype therapy is the targeting and elimination of only malignant cells, causing little or no damage to normal B-cells, thus lowering risks of infection or other immunosuppressive states. The issue then arises, what constitutes a ‘minimal’ idiotype? In our study we have focused on the CDR3 sequence of the heavy chain because of its position as the unique feature of clonal B-cells [10]. Most antibody CDRs are encoded by germline sequences with the exception of CDR3 of the heavy chain, which is produced by VDJ recombination [10]. In our strategy, we have chosen to include the CDR3 sequences from both the heavy and light chains of the MEC1 target. The CDR3 heavy chain sequence was chosen for its unique sequence while the CDR3 of the light was included to replicate as far as possible the structure of the interface between these two CDRs.

Here we outline a proof of concept approach, using sequence data from a B-cell tumor, to construct a suitable ‘target’ for phage selection and, from there, to generate antibody-based therapeutics targeting the idiotype of the tumor sIg. From cDNA sequence data from the MEC1 tumor line, CDR3 regions of the variable portions of the heavy and light chains of the sIg were identified using a database enquiry at www.imgt.org. Next these sequences were used as a target for phage selection. Because the 4D5 scFv was reported as a robust candidate for CDR grafting [17], we chose to incorporate MEC1 CDR3 sequences into this backbone (Fig. 1). To produce a mimetic of MEC1 CDR3s, the 4D5-MEC protein was directed to the periplasm of E. coli where it, apparently, folded correctly and allowed for exposure of CDR3 loops. Phage display and selection were used to enrich for binders from the Tomlinson I Library of human scFvs. Non-specific binding was negatively selected by incubating phage with the 4D5 parental scFv (also called the ‘scaffold’). After bulk enrichment for binders, we used a phage ELISA to evaluate individual reactive phage; requiring candidates to demonstrate at least a 2-fold binding preference for MEC1-CDR3s over the control 4D5-WT sequences.

Phage binding was characterized further via flow cytometry on intact MEC1 cells. Initially five phages (binders 1, 5, 7, 9 and 10) that exhibited different patterns of reactivity were considered (Fig. 4). Because phage can be ‘sticky’ and demonstrate non-specific reactivity, we cloned and sequenced the scFv insert from the five binders with the goal of first determining if the phage were unique and then generating antibody-based therapeutics.
Diversity was introduced into the Tomlinson I library via NNK randomization at 18 residues, located in and near the CDR2 and CDR3 segments of both the heavy and light chains. Therefore, the scFv inserts of the five binders (1, 5, 7, 9 and 10) were sequenced and compared one to the other (see Fig. 3 and Supplemental Fig 3). Most sequence variants were a direct consequence of the Tomlinson library mutational strategy (represented in yellow highlight, Fig. 5). However, we did note two stop codons (phages 5 and 10) and a leucine to histidine variant at position 180 (phages 5 and 7) Fig. 3.

Two of the five, binders 1 and 7, were characterized in detail. Binders 5 and 10 had reacted in an anomalous way with only ~50% of cells and were not studied further. Binder 9 was not pursued because it displayed the weakest binding. Binders 1 and 7 were cloned from their respective phage and converted into soluble proteins. These included immunotoxins and intact antibodies. However, the same general cloning approach could have produced antibody-drug conjugates, BiTE-like therapeutics or CAR T cells. Each of these technologies represents a distinct path to developing a therapeutic molecule, but all rely on the initial selection of a reactive phage to the patient’s idiotype.

We report here that immunotoxins derived from binders 1 and 7 reacted with and killed MEC1 cells and not other B-cell tumor lines, such as JVM13 or Nalm6, confirming reactivity and selectivity (Fig. 5). Further, when we cloned the heavy and light chains of the scFvs into a full-length human IgG1 vector, at least one of the antibodies, binder 1, retained binding and specificity for MEC1 cells (Fig. 6). When converted into a full-length antibody, binder-7 scFv did not exhibit any cell-binding activity. The failure to convert the binder-7 scFv into a full-length functional antibody was not investigated further but could be due technical issues or a low affinity for the cell-associate sIg. Further, we did note that binder-7-PE38 immunotoxin did not bind as robustly to MEC1 cells as did the binder-1-PE38 immunotoxin. So, it is possible that binder 7 has a lower affinity for the MEC1 idiotype.

One interpretation of the idiotype concept is that each patient has a unique sequence and all attempts to target this sequence would constitute a ‘custom-built’ therapeutic. But in many B-cell malignancies there are specific families of rearranged immunoglobulin genes that associate with disease and these may be functional in response to antigens. In CLL, for example, there are well-known gene families that arise frequently and are categorized as so-called stereotypes [26–28]. Analysis of stereotype sequences has been carried out to determine clinical outcomes and also to produce vaccine preparations [27, 29]. Thus, targeting individual tumors may be a tractable problem involving the development of some 40–50 anti-idiotype antibodies. Current approaches for the treatment of B-cell malignancies, using therapeutic antibodies to B-cell differentiation antigens, include targeting CD19, CD20 and CD22. While these antibodies, especially, those targeting CD20 have produced great clinical benefit for patients, there are on-going concerns about immunosuppression and infection. Likewise, antibodies to framework regions of sIg or light chains might provide a degree of selection but would not have the advantage of the clonal target associated with the anti-idiotype approach.

**CONCLUSION**

Using a human scFv library and phage display, it was possible to select phage for a target sequence corresponding to the CDR3s of the sIg expressed by the MEC1 tumor line. Two phages, with reactivity for intact MEC1 cells, were converted to soluble proteins that retained binding and selectivity. This strategy could be used to develop antibody-based therapeutic reagents that target the idiotype of B-cell malignancies.

**SUPPLEMENTARY DATA**

Supplementary Data are available at ABT online.

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