

Fusogenics: A Recombinant Immunotoxin-Based Screening Platform to Select Internalizing Tumor-Specific Antibody Fragments

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Antibody-based therapeutics play a vital role in the treatment of certain cancers; however, despite commercial success, various strategies are being pursued to increase their potency and hence improve patient outcomes. The use of antibodies to deliver a cytotoxic payload offers a promising alternative for more efficacious therapies. Immunotoxins are composed of an internalizing antibody fragment linked to a bacterial or plant toxin. Once internalized, the payload, such as *Pseudomonas* exotoxin A (PE), blocks protein synthesis and induces apoptosis. Typically, immunotoxins are developed by first isolating a tumor-specific antibody, which is then either chemically linked to a toxin or reengineered as a fusion protein. Here, the authors describe the development of Fusogenics, an immunotoxin-based screening method that selects internalizing tumor-specific antibodies using a functional assay. Selected immune library clones were characterized and shown to be selective against normal tissues and specific to tumor tissues. In summary, the Fusogenics immunotoxin platform represents a unique, single-step selection approach combining specificity and functionality to isolate novel internalizing tumor-specific antibody fragments with potential for direct clinical application in the treatment of cancer. (*Journal of Biomolecular Screening* 2011:90-100)

Key words: immunotoxin, *Pseudomonas* exotoxin A, immune library screening, *Escherichia coli*

INTRODUCTION

IN THE PAST 2 DECADES, ANTIBODY-BASED THERAPIES have played an increasingly important role in the treatment of cancer.¹ The clinical success of antibodies in oncology is due predominately to their innate specificity, which enables them to selectively target antigens on the surface of tumor cells. Tumor-associated antigens (TAAs) are modified proteins or glycolipids that contribute directly or indirectly to the uncontrolled tumor cell proliferation.^{2,3} Upon binding to TAAs, antibodies kill tumor cells via the activation of the antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) response or interfere with the biological function of tumorigenic receptors.⁴ Given the growing interest in therapeutic antibodies (Abs), standard screening platforms have been developed that permit the selection of human Abs to an isolated antigen or to tumor cells. Immunization of transgenic mice containing the human immunoglobulin repertoire and the screening of phage,

yeast, or ribosome display libraries are commonly used strategies against isolated antigen.^{5,6} Of these validated antibody discovery technologies, only hybridoma and phage libraries are suitable for screening live tumor cells. Conventional hybridoma screening represents a low-throughput approach that is only now being addressed through the automation of hybridoma culture and testing. With phage display, libraries expressing human antibody fragments are negatively selected with nonepithelial cells and then submitted to several rounds of positive panning against tumor cell lines or tumor tissues. Due to the ease and robustness of this screening procedure, multiple tumor-specific antibodies have been isolated from small phage immune libraries, large naive libraries, or wholly synthetic libraries.^{7,8} However, the selection of these screening strategies is based strictly on binding properties, implying that a second step is required to distinguish those with biological activity. Therefore, selected antibody fragments from display methods are subsequently converted into an IgG and tested in a variety of in vitro assays, such as ADCC, to select candidates with the desired biological activity.⁹

Despite the commercial success of therapeutic Abs, approaches to further improve potency such as mutation of Fc residue(s) or de-fucosylation of the carbohydrate chains are currently being explored to improve clinical benefit.¹⁰ Another approach is to develop antibody drug conjugates (ADCs) or immunotoxins that use the antibody as a vehicle to specifically deliver a cytotoxic

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Received Jul 26, 2010, and in revised form Sep 21, 2010. Accepted for publication Sep 23, 2010.

Journal of Biomolecular Screening 16(1); 2011
DOI: 10.1177/1087057110387425

payload such as a small molecule or a protein toxin to cancer cells.^{11,12} ADCs rely on linker technology to chemically conjugate the payload to the antibody, whereas recombinant immunotoxins are fusion proteins that contain a cytotoxic protein genetically linked to an antibody fragment. To be efficacious, an immunotoxin fusion protein engineered with a scFv or Fab fragment requires internalization without receptor cross-linking. Once internalized, bacterial toxins such as *Pseudomonas* exotoxin A (PE) and plant toxins such as bouganin, gelonin, or saporin prevent protein synthesis leading to cell death.¹² As a consequence of their cytotoxicity to mammalian cells, immunotoxins are generally expressed in microbial cells and purified either from inclusion bodies or the periplasmic space. The clinical benefits of immunotoxins have been demonstrated mostly with hematological cancers and in the case of solid tumors using local delivery strategies.^{13,14} Thus, given the need to select more potent cancer therapeutic drugs, a possible strategy is to develop a screening approach using an immunotoxin format that has the potential to simultaneously identify tumor-targeting antibodies and rapidly assess their cytotoxicity in a single step without the need for molecular reengineering.

In this study, we investigated the immunotoxin concept in a soluble screening format to select and identify novel internalizing Ab candidates from immune libraries. As a proof of concept, an internalizing anti-EpCAM Fab-PE recombinant fusion protein was used to develop a functional screening assay for measuring the level of PE-induced apoptosis. Subsequently, the concept was extended to a screening platform—namely, Fusogenics—which selects internalizing, tumor-specific Ab fragments from a Fab-PE immune library created from the plasma B cell population of colon cancer patients. The biological characterization, tumor reactivity, and cytotoxic potential of 1 clone—namely, VB6-314-PE—are used to demonstrate the potential of this novel screening approach.

MATERIALS AND METHODS

Cell culture

All tumor cell lines were purchased from ATCC (Manassas, VA) with the exception of CAL-27, which was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Cell lines were cultured in a humidified incubator at 37°C in presence of 5% carbon dioxide and maintained in their respective medium as per the provider's recommendations.

Fusogenics immune library construction

Lymphocytes, sourced from lymph nodes of colon cancer patients (Cureline, San Francisco, CA), were suspended in 1 mL Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and plasma B cells

negatively selected in the presence of 250 ng and 50 ng of antihuman IgD (BD Biosciences, Mississauga, Ontario, Canada) and antihuman Thy-1 (Serotec, Raleigh, NC) biotinylated antibodies, respectively. After 90 min on ice, cells were centrifuged and resuspended in 1 mL DMEM, 10% FBS. An equal volume of magnetic beads coated with streptavidin was added to the cells, and after 1 h on ice, naive B cells and T cells were separated magnetically and the media containing enriched plasma B cells collected.

mRNAs from enriched plasma B cells were extracted using the Oligotex™ kit (Invitrogen, Toronto, Ontario, Canada) and the first-strand cDNA synthesized using the SuperScriptIII reverse transcriptase kit (Invitrogen). Library clones were expressed in *Escherichia coli* supernatant as a Fab recombinant fusion protein comprising an antibody Fd fragment genetically linked to a truncated form of *Pseudomonas* exotoxin A, PE₂₅₂₋₆₀₈. Both chains, preceded by a PelB leader sequence, were cloned into the pING3302 plasmid (Xoma, Berkeley, CA) as a dicistronic unit under the control of an arabinose promoter. The V_L kappa repertoire was amplified by PCR with a mixture of 5' primers corresponding to the different subclasses and containing the *Sfi*I restriction site, a 6xHis tag for purification, and a 3' end constant kappa chain primer containing a *Xho*I restriction site. The PCR product was digested with *Sfi*I and *Xho*I restriction enzymes and ligated into the EcoRI-ApaI-C_H-PE-PelB-SfiI-XhoI/pING3302 plasmid (Fig. 1A). EasyShock™ 10B electrocompetent cells (BioRad, Mississauga, Ontario, Canada) were then electroporated with the purified ligation reaction (Zymo Research, Burlington, Ontario, Canada). The V_H gamma repertoire was obtained with a mixture of 5' primers and a 3' constant gamma chain domain primer containing the *Nco*I and *Apa*I restriction site, respectively. The V_H PCR product was linked to the EcoRI-PelB leader sequence into an intermediate plasmid using the *Nco*I and *Apa*I restriction sites. The full insert was digested with *Eco*RI and *Apa*I restriction enzymes and randomly cloned into C_H-PE-PelB-V_L-C_L/pING3302 plasmids (Fig. 1B). The ligation reaction and transformation in 10B cells was performed as previously described. The final recombinant plasmids containing randomly paired variable domains were then electroporated into JM109 and 10F *E. coli* strains (Zymo Research; Invitrogen).

To create the anti-EpCAM Fab-PE fusion protein—namely, VB6-845-PE—the 5' *Nco*I–3' *Apa*I restriction sites of the variable heavy chain 4D5MOCB were introduced by PCR reaction using oligonucleotide primers.¹⁵ The conserved kappa constant domain and the 5' *Sfi*I–3' *Xho*I restriction sites were added to the 4D5MOCB variable light chain by splice overlapping extension PCR.

Fusogenics immune library screening

Transformed *E. coli* JM109 cells were plated onto LB-agar Q-tray plates (22 × 22cm) supplemented with 25 µg/mL of

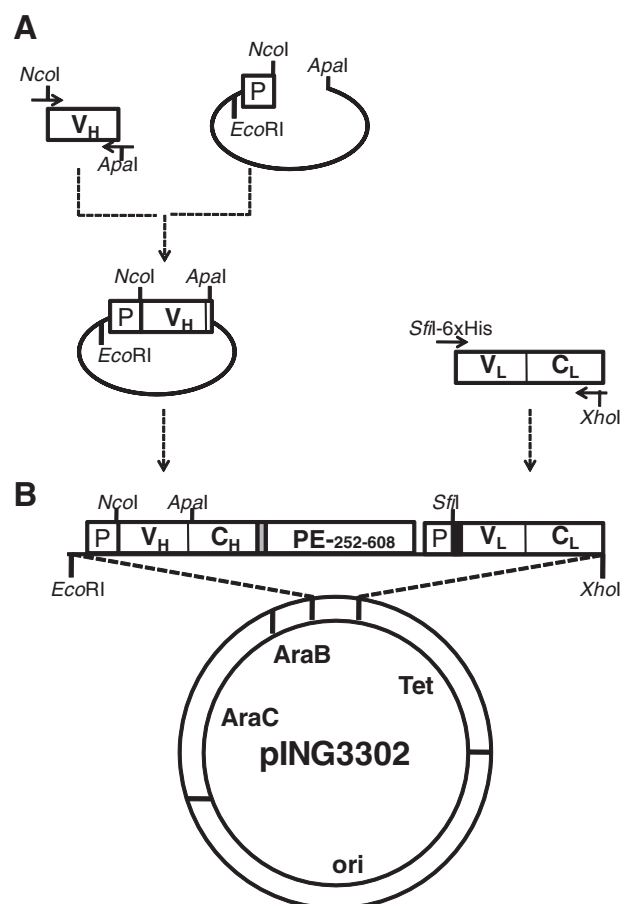


FIG. 1. Schematic representation of Fusogenics immune library construction. V_H , V_L , C_H , C_L , PE, and P abbreviations correspond to the variable heavy and light chain and heavy and kappa chain conserved domains and truncated form of *Pseudomonas* exotoxin A from amino acids 252 to 608, to the PelB leader sequence, respectively. (A) The V_H and V_L repertoires were generated by PCR using oligonucleotide primers with their respective restriction enzymes to facilitate cloning. The V_H repertoire was inserted in front of a PelB leader using the *NcoI* and *ApaI* restriction enzymes. (B) The V_H and V_L repertoires were randomly cloned into the pING3302 plasmid using *NcoI*-*ApaI* and *SfiI*-*XhoI* restriction enzymes, respectively. The flexible linker and 6xHis tag are indicated by gray and black shading, respectively. The location of the bacterial origin of replication (*ori*), the tetracycline resistance gene (*tet*), the arabinose-inducible sequence encoding the *araC* protein and *araB* promoter, and the *EcoRI* and *XhoI* restriction sites of the pING3302 are indicated.

tetracycline. To maximize the efficiency of the screening procedure, single colonies were picked with a colony picker (Genetix, New Milton, Hampshire, UK) and all liquid transfers performed with a BiomekFX (Beckman Coulter, Fullerton, CA). Briefly, 96-well plates containing 150 μ L of 2xYT supplemented with 25 μ g/mL of tetracycline were inoculated with a single colony using automated picking and incubated overnight at 37°C in a humidified shaking incubator (Liconic Instruments, Woburn, MA).

Overnight incubation allowed all clones to reach the growth phase plateau, thereby standardizing the inoculums used for the induction plate. A 20- μ L culture from each well was transferred into a 96-well daughter induction plate containing 130 μ L of terrific broth (TB) and incubated at 37°C with constant shaking. Then, 20 μ L of 50% sterile glycerol was added to each well of the 2xYT plates, which were then stored at -20°C. After 7 h of incubation, the TB plates were induced with 0.2% L-arabinose and incubated overnight at 25°C. After centrifugation at 5000 rpm for 30 min, 10 μ L of supernatant was added to 96-well FMAT plates preseeded with SW-480 colon tumor cells at a density of 8000 cells per well (in DMEM supplemented with 1% penicillin-streptomycin) and incubated overnight in a 5% CO₂ incubator set at 37°C. The remaining supernatant was stored at -20°C in 96-well plates for further analysis. Using the 8200 Cellular Detection System (Applied Biosystems, Streetville, Ontario, Canada), the percentage of apoptotic cells was measured by the addition of 20 μ L of Annexin V buffer (140 mM NaCl, 12.5 nM CaCl₂, and 10 mM HEPES) containing Annexin V coupled to Alexa-647 (Invitrogen) diluted 1/3000 and 0.75 nM CentriRed (Applied Biosystems) for 50 min at room temperature (RT). *E. coli* JM109 cells transformed with either VB6-845-PE or the pING3302 plasmid alone were used as positive and negative controls, respectively. Wells showing a level of apoptosis of at least 2-fold higher than the negative control were retested in 3 independent wells to demonstrate reproducibility. To finalize the selection, candidate clones were then retested in triplicate against SW-480 colon tumor cells and the B cell lymphoma, CA46.

Dot-blot and enzyme-linked immunosorbent assay quantification

To determine the percentage of expressing immune library clones, 50 μ L of induced supernatant from randomly chosen immune library clones was transferred onto a nitrocellulose membrane using a mini-fold apparatus. After blocking with a 1 \times Tris-buffered saline (TBS) 3% bovine serum albumin (BSA) solution for 1 h, the membrane was incubated with an antihuman kappa horseradish peroxidase (HRP) conjugate (Sigma-Aldrich, St. Louis, MO) (1/1000) for 2 h and bound antibody detected using DAB solution (Pierce, Rockland, IL).

To quantify the level of expression of immune library clones, a 96-well plate (Immulon 1B) was coated with 10 μ g/mL of rabbit anti-*Pseudomonas* exotoxin A (Sigma-Aldrich) overnight at 4°C and blocked with 1% BSA for 1 h at 22°C. Induced supernatants diluted from 1/320 to 1/2560 were added to the plate and incubated for 2 h at 22°C. To detect bound Fab-PE fusion protein, an antihuman kappa light chain HRP conjugate (Sigma-Aldrich) was added to each well and, after 1 h at 22°C, the reaction developed with 3,3',5,5'-tetramethylbenzidine. A standard curve was obtained with purified VB6-845-PE and induced pING3302 supernatant used as a negative control.

Expression and purification of immune library clones

Fermentation of immune library clones was performed in a 15-L CHEMAP fermenter using TB medium. Cultures were induced at an OD₆₀₀ of 2 with a mixture of feed (50% glycerol) and inducer (200 g L-arabinose). At 24 h postinduction, the culture was harvested, centrifuged at 8000 rpm for 30 min, concentrated, and diafiltered against 20 mM sodium phosphate (pH 7.0). The supernatant was then applied to a charged chelating sepharose column and the column washed with 10 mM imidazole. Bound Fab-PE was eluted with 250 mM imidazole. To obtain purity >90%, the eluate of the chelating sepharose column was applied onto a size exclusion column S200 equilibrated with 20 mM sodium phosphate, 150 mM NaCl, pH 7.5. Purity following the size exclusion column was confirmed by colloidal blue staining and identity by Western blotting using rabbit anti-*Pseudomonas* exotoxin A (Sigma-Aldrich) followed by goat antirabbit HRP conjugate (The Binding Site, San Diego, CA).

Sequence analysis

Nucleotide sequence of the heavy and light chain variable domains was performed using the GenomeLab™ Dye Terminator Cycle sequencing (Beckman Coulter, Mississauga, Ontario, Canada) with specific primers. Sequences were analyzed using NCBI IgBlast to determine CDR regions and V gene usage.

Clone characterization and selection

Cell surface reactivity and internalization. The reactivity of immune library clones was determined against a panel of tumor cells representative of cancer indications by flow cytometry using a FACS Calibur (BD Biosciences). Briefly, tumor cells (1×10^6 /mL) were incubated with 10 µg/mL of purified Fab-PE for 2 h on ice. After washing away the unbound material, bound Fab-PE was detected using a biotinylated goat antihuman H&L chain antibody (Pierce) followed by Cy-5-streptavidin (Pierce). Cells were analyzed on a FACS Calibur following propidium iodide (Molecular Probes, Eugene, OR) staining. The rate of internalization was determined by measuring the Fab-PE protein bound to SW-480 tumor cells after incubation at 37°C for 15, 30, 60, and 120 min. The results were presented as percent decrease in median fluorescence (MF) versus a 4°C sample (100%).

Affinity. The functional affinity of individual clones was determined by flow cytometry.¹⁶ Briefly, MDA-MB-435S cells (6×10^5 /mL) were incubated with increasing concentrations of Fab-PE for 2 h at 4°C to establish a saturation curve. Bound Fab-PE was detected as described above. The functional affinity, expressed as the dissociation constant K_D , was calculated by the Lineweaver-Burk method of plotting the inverse of the

MF as a function of the inverse of the antibody concentration. The dissociation constant was determined by the following equation: $1/F = 1/F_{\max} + (K_D/F_{\max})(1/[Ab])$, where F corresponds to the background subtracted MF, and F_{\max} was calculated from the plot.

Potency. The potency was measured by an MTS assay (Promega, Madison, WI). Briefly, SW-480 and CA46 cells were seeded at 5000 cells per well and incubated at 37°C for 3 h. Purified Fab-PE was added to the cells over a range of concentrations, incubated for 3 days, and an IC₅₀ interpolated from the resulting plot.

Tumor and normal tissue reactivity. The selectivity and specificity of Fab-PE fusion proteins was assessed against critical normal and tumor tissues using immunohistochemistry. Briefly, formalin-fixed tissues slides (TriStar, Rockville, MD) were deparaffinized in xylene, hydrated in alcohols, and blocked for endogenous peroxidase with 3% hydrogen peroxidase. For antigen retrieval, slides were heated to 125°C in target retrieval solution (Dako Diagnostics, Mississauga, ON) in a decloaking chamber. Fab-PE was then added to the tissues and incubated for 1 h at RT. After washing, bound Fab-PE was detected with rabbit anti-*Pseudomonas* exotoxin A (Sigma-Aldrich) followed by EnVision-HRP (Dako Diagnostic) and cytomation liquid DAB plus substrate chromogen system (Dako Diagnostic). Slides were counterstained in Harris hematoxylin (Fisher, Ottawa, Ontario, Canada), coverslipped using cytomation mounting media (Dako Diagnostic), and analyzed for cell surface staining by a board-certified pathologist.

RESULTS

Fusogenics: screening parameters

The feasibility of this approach, and in particular the expression level in *E. coli* supernatant and screening parameters, was tested using VB6-845-PE, an internalizing anti-EpCAM Fab fragment genetically linked to PE₂₅₂₋₆₀₈. To select the optimal electro-competent *E. coli* strains for supernatant secretion, 10F and JM109 cells were transformed with the recombinant plasmid. The level of expression was assayed in either a 96-well plate or 96-well deep plate and compared to those typically obtained under shake-flask conditions. Western blot analysis showed that VB6-845-PE expression level in induced *E. coli* supernatants was similar with either type of 96-well plate but, as expected, lower when compared to shake-flask levels (**Fig. 2A**). With respect to host strain expression, VB6-845-PE expression was higher in JM109 supernatant (~2.5 µg/mL by enzyme-linked immunosorbent assay [ELISA]) as compared to 10F, thus making JM109 the preferred host cell.

Inhibition of *E. coli* growth during the functional assay was a prerequisite to ensure a readable outcome. Therefore,

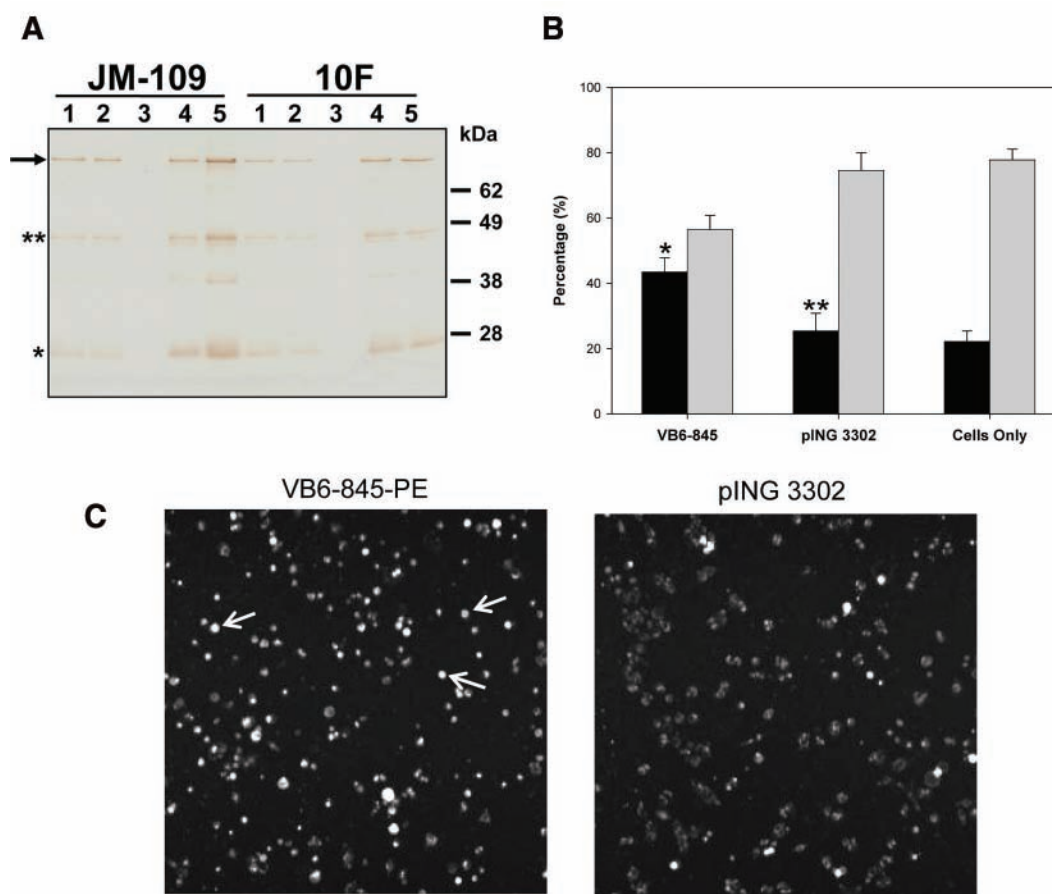


FIG. 2. Expression and cytotoxicity of soluble VB6-845-PE expressed in *Escherichia coli*. (A) Western blot analysis of VB6-845-PE. Induced supernatant of VB6-845-PE in JM109 or 10F *E. coli* strains from 96-well plate (lanes 1 and 2), deep-well plate (lane 4), shake-flask (lane 5), and pING3302 from 96-well plate (lane 3) were loaded under nonreducing conditions and immunoblotted with a goat antihuman kappa antibody coupled to horseradish peroxidase (HRP). The arrow indicates the full-length 89.4-kDa VB6-845-PE fusion protein. Single and double asterisks indicate the light chain and Fab moiety of VB6-845-PE, respectively. (B) Percent of apoptotic cells (black bar) and live (gray bar) SW-480 cells, measured with Annexin V staining and CentriRed, respectively, was determined after 24-h incubation with VB6-845-PE or pING3302 JM109 *E. coli*-induced supernatant. *Cells incubated with VB6-845-PE showed a significant increase in apoptotic cells as compared to pING3302-treated cells (Student *t* test, $n = 10$, $p = 0.018$). **No significant difference (Student *t* test, $n = 10$, $p = 0.613$) was observed between pING3302-treated cells and cells alone. (C) Representative wells with live and apoptotic SW-480 cells after 24-h incubation with VB6-845-PE or pING3302 JM109 *E. coli*-induced supernatant. White arrows indicate Annexin V-positive stained cells.

preliminary experiments performed in a 96-well plate showed that a mixture of 10 to 30 μL of JM109-induced supernatants with 100 μL of DMEM containing antibiotics was *E. coli* free for up to 24 h at 37°C. Therefore, the cytotoxicity of 10, 20, and 30 μL of VB6-845-PE and pING3302 JM109-induced supernatants was measured using the EpCAM-positive tumor cell line SW-480 and an EpCAM-negative B cell lymphoma, CA46, seeded on a FMAT plate (Fig. 2B,C). After 24-h incubation, apoptotic and viable cell numbers, in the presence of 10 μL pING3302 supernatant, were comparable to untreated control wells. In contrast, VB6-845-PE supernatant led to a decrease in viable cell number and a correspondingly significant increase

in apoptotic cells, suggesting that the VB6-845-PE fusion protein, present in the *E. coli* supernatant, was biologically active. No significant toxicity was observed with CA46 cells, demonstrating the specificity of VB6-845-PE. To explore the lower limit of the readout, the assay was repeated in the presence of 10 μL of 10- to 1000-fold diluted supernatant. Only the 1000-fold dilution was less cytotoxic (data not shown). Based on the ELISA data, a 1000-fold diluted VB6-845-Fab-PE concentration was below the IC_{50} obtained with the purified protein explaining the lower cytotoxicity. Of note, nonspecific cytotoxicity was detected with both cell lines in the presence of 20 or 30 μL of pING3302 supernatant, suggesting that higher

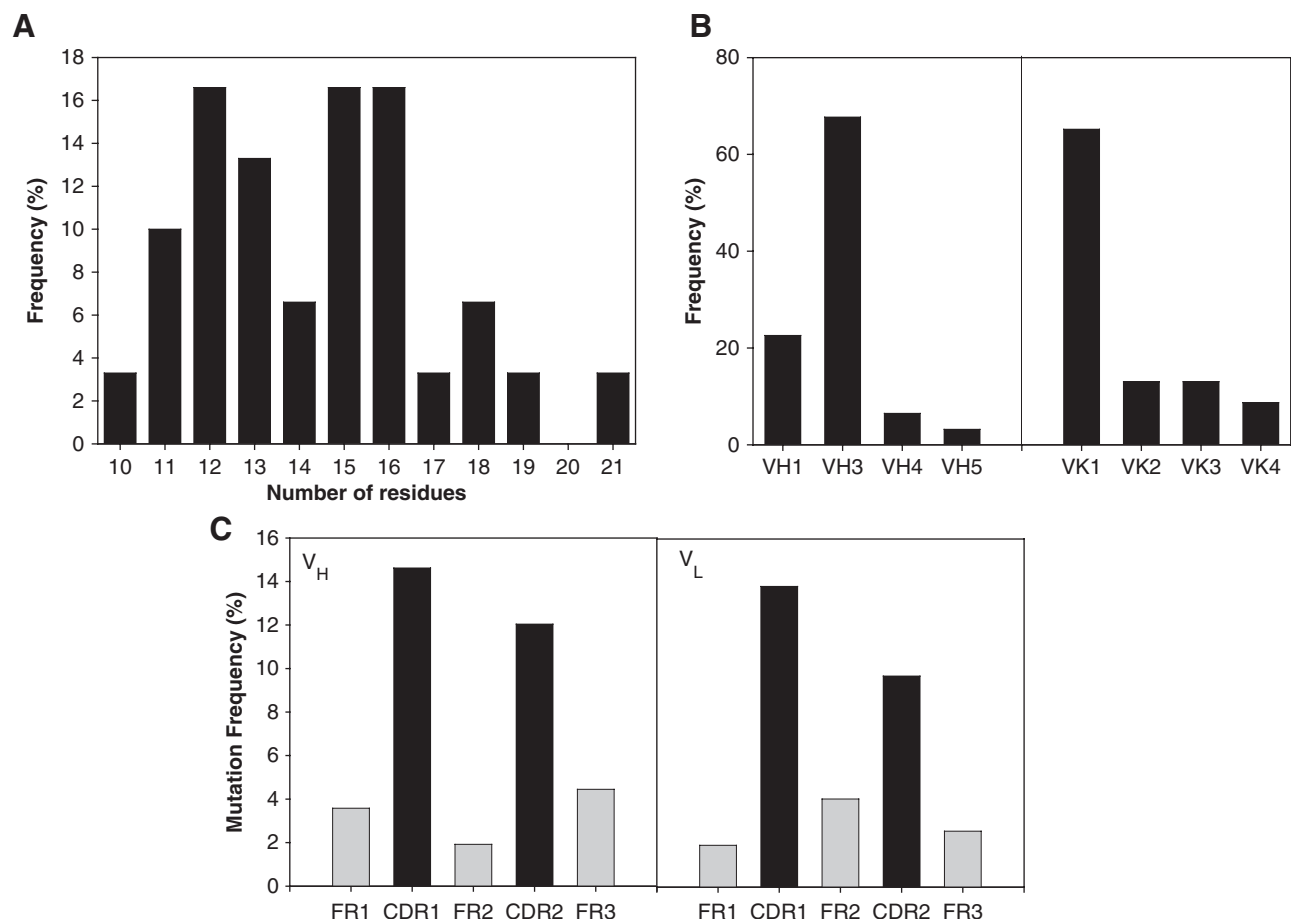


FIG. 3. Sequence analysis of immune library clones. **(A)** Frequency of V_H CDR3 length of Fab-PE immune library clones. **(B)** Frequency of V-gene usage for the variable heavy and light chains. **(C)** Mutation frequency in the V_H (left) and V_L (right). Mutations in the FR1, FR2, and FR3 (gray bars) and CDR1, CDR2, and CDR3 (black bars) of the V_H and V_L domains were identified by alignment with germline sequences and normalized to the length of the FR and CDR regions (for V_H , FR1 = 29, FR2 = 14, FR3 = 32, CDR1 = 5, CDR2 = 17; for V_L , FR1 = 20.5, FR2 = 15, FR3 = 32, CDR1 = 11, CDR2 = 7). CDR3 and FR4 regions were not included in the analysis.

volumes are toxic to the cells. These data demonstrate that the detection of soluble Fab-PE fusion protein is measurable and that the assay is sensitive over a range of expression levels using the 96-well plate format.

Fusogenics: immune library creation

To enrich the immune library with tumor-reactive antibody fragments, only plasma B cells isolated from the lymph nodes of 3 colon cancer patients were used as starting material. After mRNA extraction from 2.6×10^6 plasma B cells, only IgG-derived V_H fragment and V_L kappa repertoires were amplified by using an antisense oligonucleotide primer homologous to the 5' end constant domain. A library representing a repertoire of Fab genes was then assembled as a discitronic unit using a V_H and V_L cloning cassette. The size of the library, generated from 4 independent electroporations using a single ligation reaction, was estimated at 500,000 unique clones. The dot-blot

analysis of random colonies grown and induced in a 96-well plate showed that approximately 70% of the clones produced a soluble Fab-PE fusion protein in the supernatant (data not shown). A third of the clones had an expression level estimated at approximately 2.5 $\mu\text{g/mL}$, another third ~ 1 $\mu\text{g/mL}$, and the remaining below 0.5 $\mu\text{g/mL}$, but they were all within the functional range of the assay. The diversity of the library was evaluated by analyzing the sequences of 30 expressing clones. The sequence analysis revealed 30 unique sequences, and the CDR3 length of the variable heavy chains showed a distribution between 10 and 21 amino acid residues (**Fig. 3A**). Different heavy and light gene families were represented in the library; however, more than 65% of the clones had either V_H3 or V_K1 subclasses (**Fig. 3B**). As well, alignment of the variable domains with germinal sequences revealed that 88% of the heavy and light chains contained on average 5.5% and 4% mutated residues, respectively, corresponding to somatic mutations. As expected, the number of mutations in the CDR1 and

CDR2 loops was greater by 2- to 3-fold compared to framework (**Fig. 3C**).

Immune library screening

The immune library was screened against SW-480 tumor cells that had been detached using PBS-EDTA, to avoid proteolytic degradation of potentially interesting cell surface antigens. Following the second round of positive selection, ~1.7% of the clones had an apoptotic level that was at least 2-fold higher than the pING3302 negative control. Of these, only 4.2% were cytotoxic to SW-480 but not CA46, suggesting specificity toward epithelial markers and resulting in an overall success rate of 0.07% for the screening procedure. To prioritize selected clones, binding to SW-480 cells was measured by flow cytometry using induced supernatant (data not shown). Clones with the highest binding activity were purified and tested against SW-480 and CA46 cell lines. As an illustration, VB6-314-PE clone is shown (**Fig. 4A,B**). VB6-314-PE bound to SW-480 cells and an IC_{50} of 300 pM was measured. In contrast, no binding or IC_{50} was obtained against CA46 cells. As well, SW-480 cells incubated at 37°C showed that more than 60% of bound VB6-314-PE was internalized within an hour (data not shown). These data demonstrated that internalizing Fab-PE clones with specific cytotoxicity toward the SW-480 cell line can be isolated using the Fusogenics screening approach.

Biological characterization of selected clones

To assess the selectivity of prospective clones for tumor versus normal, the antibody fragment was tested on a low-density (LD) array of critical normal tissues by immunohistochemistry. If minimum to no cell surface staining was observed, then the antibody fragment was tested in a high-density (HD) formalin-fixed tumor microarray. Subsequently, a panel of tumor cell lines was tested by flow cytometry to confirm that the cognate antigen is also present on established cell lines. As illustrated with VB6-314-PE, only minimal cell surface staining on critical normal tissues was observed with lung, colon, and stomach (**Table 1**). However, in these instances, the maximum score for membrane staining is only 1+ with no more than 20% of cells showing positive staining. In contrast, VB6-314-PE reactivity was detected on all tumor types tested but in particular kidney, head and neck, and prostate tissues (**Table 2**). The intensity and percent tumor membrane staining increased compared to critical normal, suggesting a marked overexpression of the antigen. For most tumor tissues, the 2+ staining also corresponded to an increased percentage of membrane-stained cells. It is noteworthy that, although lung tumor tissue exhibited a greater percentage of cells with more intense membranous staining, the frequency of positive cores was less than observed in the normal lung panel. The higher frequency of normal lung tissue cores being stained may be explained by the

unique architecture of lung tissue. The porous appearance of normal lung tissue makes the core susceptible to nonspecific staining commonly seen along tissue edges. Conversely, the cellular aggregation of the epithelium in lung tumor tissue precludes an edge effect; hence, fewer samples show positive staining.

Flow cytometry demonstrated that VB6-314-PE was reactive to most tumor cell lines tested. The strongest binding was to head and neck (SCC-25), pancreatic (BxPC-3), and breast (MDA-MB-435S) tumor cells, closely mirroring the pattern of reactivity observed with tumor tissue (**Table 3**). The dissociation constant of VB6-314-PE was determined by flow cytometry using MDA-MB-435S cells and calculated to be 2.9×10^{-8} M (data not shown). However, this number may represent an underestimation as the same method measured a K_D for VB6-845-PE that was almost 10 times lower than that obtained by BIAcore analysis.^{15,16}

DISCUSSION

This report describes the development of the Fusogenics screening platform in which a Fab-PE immunotoxin format was used to select internalizing tumor-specific Ab fragments using a functional cytotoxic assay. The screening parameters were established with an internalizing anti-EpCAM Fab-PE fusion protein as a proof of concept. Subsequently, a Fab-PE immune library was created, and the specificity and selectivity of the process was illustrated with the biological characterization of clone VB6-314-PE.

The concept of the Fusogenics approach is based on the direct selection of internalizing Fab fragments. The Fab format was chosen over the scFv because of the inherent stability provided by the mutual interaction across the interface of the V_L/V_H and C_L/C_H domains and by the interchain disulphide bond.¹⁷ Furthermore, many scFv fragments derived from natural antibodies are prone to dimerization and aggregation due to an exposed hydrophobic patch of amino acids normally buried in the interface of the variable and constant domains.¹⁸ Dimerized or aggregated scFvs could trigger cell surface receptor internalization via cross-linking, leading to the selection of false positives. Therefore, the stability of the Fab ensures the selection of Ab fragments that do not require target dimerization for internalization. For the toxin moiety, the highly potent truncated form of PE was an ideal choice as only few molecules are required to kill a tumor cell. Since PE induces apoptosis by preventing protein synthesis, the readout of the Fusogenics approach was to measure an apoptotic index.¹³ Although only a Fab-PE format has been tested, it is conceivable that any ligand, encompassing a stable antibody format or scaffold protein, could be engineered as a soluble fusion-PE protein. Similarly, the PE moiety could be changed to other types of effector proteins as long as they trigger a measurable biological effect.

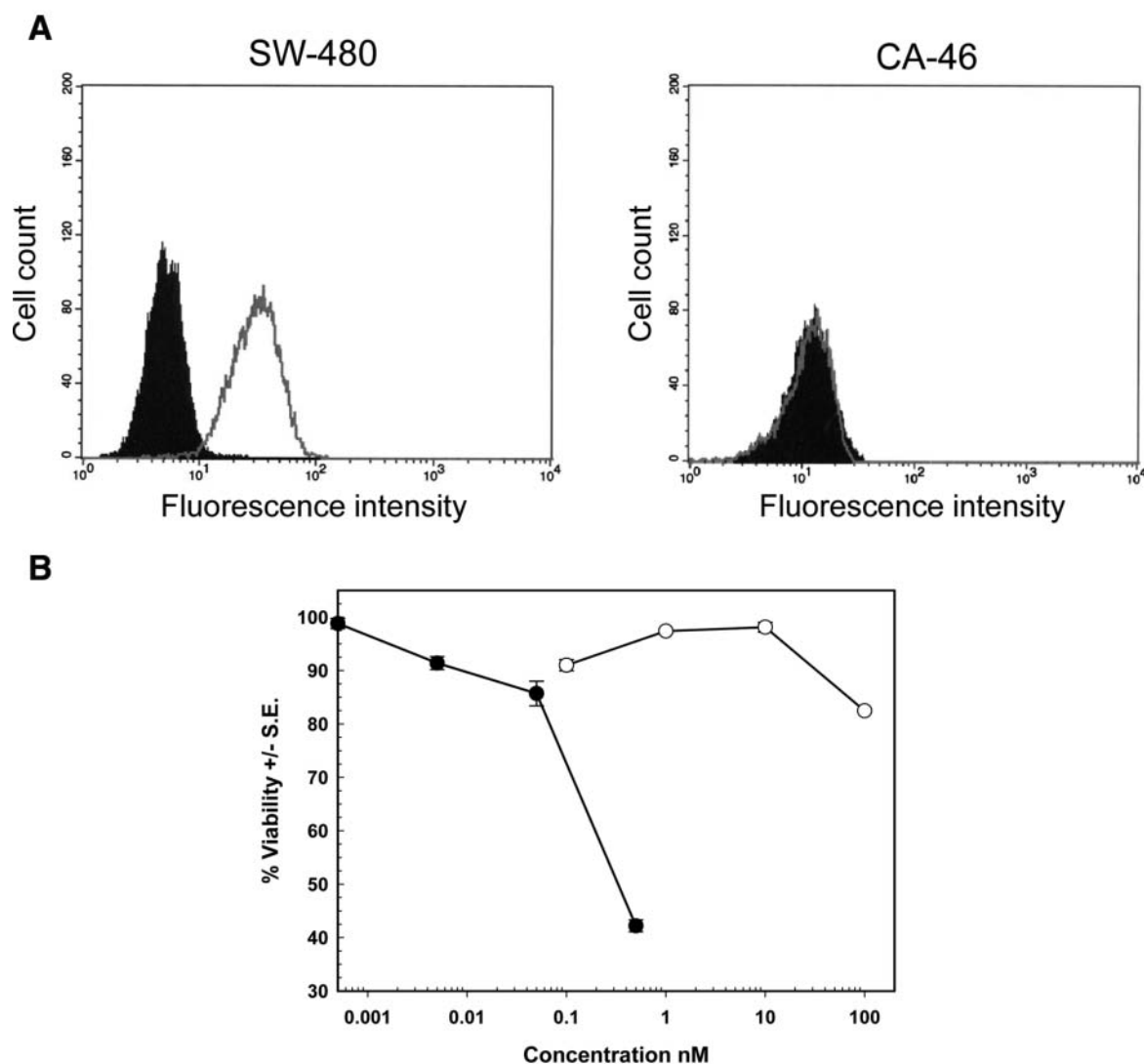


FIG. 4. Biological activity of a purified, representative clone from the immune library. VB6-314-PE at 10 $\mu\text{g}/\text{mL}$ was mixed with SW-480 or CA46 cells for 2 h at 4°C. Bound VB6-314-PE was measured by flow cytometry, and the filled area corresponds to the negative control (secondary antibody alone) and the gray line to VB6-314-PE specific binding. (**B**) Purified VB6-314-PE was added to SW-480 reactive tumor cells (filled circle) and CA46 cells (open circle) in triplicate. After 3 days of incubation, MTS reagent was added and the IC_{50} determined. These data are representative of 3 independent experiments.

Compared to other antibody discovery platforms, the Fusogenics approach does not permit a rapid de-convolution of the library for binder enrichment. To address this issue, 3 parameters were considered to minimize the size of the library without compromising its diversity. First, the library was created from enriched plasma B cells and thus focused only on antibody-producing B cells. Second, plasma B cells were obtained from regional draining lymph nodes of cancer patients with the intent of selecting for tumor antigen-driven B cells, thereby increasing the probability of identifying unique antibody fragments specific to cell surface TAAs.¹⁹ Third, IgMs were omitted from the library by using IgG-specific primers

during the PCR amplification. Combining these 3 characteristics created an enriched and diverse library of tumor-reactive clones.

One-third of the clones did not express a full-length Fab-PE fusion protein probably due to the mal-folding during heavy and light chain pairing, unfavorable codon usage, or stop codons introduced by the PCR reactions. Similar observations have been reported in particular with phage display where poorly folded scFv or Fab fragments yielded low numbers of phage, which are subsequently subtracted in favor of better producers during the panning selection. It is known that the coexpression with periplasmic chaperones could facilitate the

Table 1. Critical Normal Tissue Microarray

<i>Tissue</i>	<i># Stained Cores</i>	<i>Intensity and (%) Tumor Membrane Staining</i>
Brain	0/10	NA
Colon	2/5	1 + (5-10)
Lung	5/10	1 + (10-20)
Heart	0/9	NA
Stomach	2/4	1 + (10)
Pancreas	0/10	NA
Liver	0/10	NA
Kidney	1/10	1 + (10)

Scoring was evaluated on a 0 to 3 + scale, with 0 equal to no or traces being less than 1 + membrane staining. Grades 1 + to 3 + represent increased intensity of staining, with 3 + being strong, dark brown staining. Values in parentheses indicate the highest percentage of cells stained in the scored range. NA, not applicable.

Table 2. Tumor Tissue Microarray

<i>Tissue</i>	<i># Stained Cores</i>	<i>Intensity and (%) Tumor Membrane Staining</i>
Lung	2/12	2 + (50-70)
Breast	4/11	1 + (5-10)
Ovary	6/12	1 + (5-20)
	1/12	2 + (80)
Colon	1/12	1 + (5)
	3/12	2 + (10-30)
Melanoma	4/10	1 + (5-10)
	1/10	2 + (50)
Pancreas	2/12	1 + (10)
	2/12	2 + (10-50)
Kidney	3/12	1 + (10-60)
	7/12	2 + (20-70)
Head and neck	8/12	1 + (5-20)
	1/12	2 + (20)
Liver	1/11	2 + (50)
Prostate	7/12	1 + (10-30)
	1/12	2 + (50)

Scoring was evaluated on a 0 to 3 + scale, with 0 equal to no or traces being less than 1 + membrane staining. Grades 1 + to 3 + represent increased intensity of staining, with 3 + being strong, dark brown staining. Values in parentheses indicate the highest percentage of cells stained in the scored range.

folding of antibody fragments, leading to an increased level of soluble protein.²⁰ Therefore, it is conceivable that this approach could be applied to the Fusogenics concept to further increase the frequency of expressing clones.

The sequence analysis validated the immune library by demonstrating that the percentages of V_H and V_L subfamilies mirror the human B cell V-gene repertoire and was similar to that obtained from various phage libraries.⁷ As well, the evenly distributed CDR3 heavy chain length and the lack of redundancy were indicative of a diversified immune library. During an immune response, somatic mutations are introduced into rearranged Ig genes, and as a consequence, B cells with improved antibody/antigen binding properties are selected.²¹

Table 3. VB6-314 Tumor Cells Profiling Data

<i>Indication</i>	<i>Cell Line</i>	<i>Fold MF ± SE</i>
Head and Neck	SCC-25	11.85 ± 0.15
	CAL 27	7.55 ± 3.55
Pancreas	BXPC-3	11.35 ± 0.75
	MIA PaCa-2	2.0 ± 1.0
Breast	MB-435S	10.65 ± 1.75
	MB-231	1.7 ± 1.0
Colon	HT-29	10.25 ± 0.05
	SW-480	4.1 ± 1.9
Prostate	LNCaP	10.0 ± 0.1
	PC-3	2.0 ± 1.0
Lung	A-549	9.6 ± 0.2
	NCI-H460	9.05 ± 0.15
Liver	Hep-G2	5.35 ± 0.15
Ovary	SKOV-3	4.1 ± 0.8
Kidney	ACHN	3.65 ± 1.55
	A-498	2.85 ± 0.25
Lymphoma	CA-46	1.0 ± 1.0

The fold median fluorescence, MF, corresponds to the average of 2 independent experiments.

The higher somatic mutation rates, found in the CDR1 and CDR2 loops compared to the framework, implied that antibody fragments of antigen-activated B cells were included in the immune library creation. A similar increase of somatic mutations in the CDR1 and CDR2 loops was reported with a phage library using isolated splenic B cells.²²

To date, all tumor-reactive clones selected with the Fusogenics approach are nonreactive with critical normal tissues showing the efficiency of the negative selection step, which eliminated the nonspecific binders created by the random V_H - V_L pairing. It may also reflect the fact that immune tolerance minimizes the appearance of self-reactive clones, thereby favoring those clones that are immunologically driven by the disease. Half of the clones that passed CN-TMA had reactivity restricted to 2 or 3 tumor indications, whereas the remaining antibodies, including VB6-314-PE, reacted with multiple tumor types and demonstrated higher levels of membrane reactivity. Indeed, the membrane binding and internalization of VB6-314-PE was confirmed by flow cytometry using a panel of epithelial tumor cells that express the antigen in its natural conformation. The double-digit nM dissociation constant obtained with VB6-314-PE is in the range of other selected clones (data not shown) and corresponds to the trend observed with phage immune libraries as the innate immune response generally yields antibodies with an average affinity. Xenograft studies have shown that scFvs with nM affinities are optimal for accumulation in a tumor, whereas higher affinities can actually impede diffusion into the tumor.²³ In addition, other factors such as the nature of the target, its density, and rate of internalization also affect the potency of an immunotoxin and hence in vivo efficacy.^{24,25} Therefore, the need for an affinity maturation step will

be determined on an individual basis, being guided by the preliminary data obtained from in vivo efficacy studies using tumor xenografts. In the event that affinity maturation is required, libraries containing randomized targeted CDR mutations will be engineered, and using the Fusogenics screening method, clones with higher cytotoxicity will be selected as a surrogate for improved affinity. This approach was validated with one of our preclinical candidates showing a 7-fold increased binding affinity (manuscript in preparation). Of note, the immunogenicity of the PE bacterial protein has limited the clinical benefit of PE-containing immunotoxins.¹⁴ Therefore, for systemic applications, the final preclinical candidate will be engineered with the Fab fragment genetically linked to a variant protein of bouganin, a ribosome inactivating protein that has been de-immunized by T cell epitope depletion.¹⁶

The Fusogenics approach complements other methods that select internalizing antibodies. Indeed, a similar strategy using PE38 has been described using purified full-length antibodies.²⁶ For this method, the ZZ domain derived from *Streptococcal* protein A is linked to PE38, and the ability of the Ab-ZZ-PE38 complex to kill tumor cells is determined. Approaches using a pH-sensitive fluorescent probe such as CypHer5, coupled to an antibody, also predicted internalization.²⁷ As well, a variation on phage display has been used to isolate phage from within the endosome as a means to select internalizing antibody fragments.²⁸ This strategy led to the identification of several internalizing scFvs that bind to TAAs such as MCAM/CD146.²⁹

In contrast to conventional discovery platforms, Fusogenics can potentially isolate antibodies against any target, known or novel, as long it is seen by the patient's immune system. Not knowing the identity of the target could represent a challenge in drug development as this may raise concerns as to the appropriateness and design of toxicology studies and ultimately the safety of the molecule heading into clinical trials. To this end, we have developed a rapid proteomic approach for antigen identification based on immunoprecipitation with the antibody prior to 2D-LC separation in tandem with tandem mass spectrometry (MS/MS).³⁰

In conclusion, the Fusogenics approach permits the isolation of internalizing antibody fragments with proven cytotoxic potential. The selection stringencies are based on specificity to tumor tissues and selectivity against normal tissues, internalizing properties, and nM range affinity. And finally, since immunotoxins are generally produced by microbial expression, Fusogenics represents a streamlined development path from screening to preclinical evaluation with only a molecular reengineering step required to switch from the immunogenic PE toxin to the de-immunized bouganin payload.

ACKNOWLEDGMENTS

We gratefully acknowledge Dr. Adrian Schwartz Mittelman and Shauna Loewen for technical assistance.

AUTHOR DISCLOSURE

Jeannick Cizeau, Marianne Torres, Sharla G. Cowling, Stacy Stibbard, Arjune Premasukh, Joycelyn Entwistle, and Glen C. MacDonald are employees of Viventia Biotechnologies, Inc. who have direct financial interest in the subject matter discussed in this manuscript.

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