

An anti-GD2 single chain Fv selected by phage display and fused to *Pseudomonas* exotoxin A develops specific cytotoxic activity against neuroblastoma derived cell lines

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Abstract. Since the disialoganglioside GD2 is abundantly present on the surface of neuroblastoma cells, we constructed a new recombinant immunotoxin for possible clinical use in patients with neuroblastoma. A functional 14.18 scFv-phage was obtained by selection of an anti-GD2 hybridoma derived phage antibody mini-library on the neuroblastoma-derived, GD2-expressing cell line IMR5. By insertion into the bacterial expression vector pBM1.1 the selected scFv was fused to a deletion mutant of *Pseudomonas* exotoxin A (ETA'). Periplasmically expressed 14.18(scFv)-ETA' bound to the GD2 expressing cell line IMR5, but not to the GD2 negative Hodgkin-derived cell line L540Cy as documented by ELISA and flow cytometry. The recombinant immunotoxin (rIT) inhibited cell viability of IMR5 cells by 50% at concentrations (IC₅₀) of 0.326 µg/ml. This recombinant immunotoxin will be further investigated *in vivo* for its value as a new immunotherapeutic agent for the treatment of patients with neuroblastoma.

Introduction

Neuroblastoma is one of the most common solid tumors in children originating from neural crest cells (1). At the time of diagnosis, the majority of patients have widespread disease (clinical stage IV) with a generally poor prognosis (2). Despite intensive multimodal therapy, recurrence from

minimal residual disease (MRD) occurs in over 50% of patients (3). Thus, the selective elimination of MRD using monoclonal antibody-based immunoconjugates after standard treatment might improve the outcome in neuroblastoma.

Disialoganglioside (GD2) is extensively expressed on the surface of neuroblastoma cells, but is rare or absent on normal tissues (4). In this regard, GD2 represents a promising target for adjuvant neuroblastoma immunotherapy. The murine anti-GD2 monoclonal antibody (mAb) 14G2a (5) and its chimeric (human/mouse) variant ch14.18 (6) are being tested in several clinical trials and have shown some success in patients with resistant disease (7-9). The anti-tumor effect of antibody-therapy with ch14.18 is due to cell-mediated lysis of the neuroblastoma cells by ADCC, complement-dependent cytotoxicity (CDC) (10) and anti-idiotypic antibodies (11,12). In order to enhance their specific anti-tumor activity, anti-GD2 mAbs including ch14.18 were chemically conjugated to radioactive isotopes (13), cytotoxic drugs or protein toxins (14,15). Subsequently, recombinant immunoglobulin single chain variable fragments (scFv) were developed by new DNA technologies (16). These antibody fragments are defined, compact molecules, which can rapidly penetrate into malignant tumors (17). ScFvs can genetically be fused to drugs, cytokines (18) or to other scFvs to form bivalent or bispecific antibodies (19,20). Examples of genetically engineered fusion proteins against GD2 include a ch14.18-GM-CSF fusion protein and a ch14.18-IL2 fusion protein (21,22).

A chemically-linked ricin A-chain immunotoxin (14.18-SMPT-dgA) constructed with ch14.18 mAb exhibited powerful specific cytotoxicity (IC₅₀ of 6x10⁻¹² M) against GD2⁺ tumor cells *in vitro* curing 100% of SCID mice treated one day after tumor challenge with a single injection of 8 µg (14). Very recently, our group generated a recombinant 14.18 hybridoma-derived scFv mini-library to establish a new selection procedure on intact cell surfaces resulting in dramatically reduced numbers of unspecifically bound scFv-free phages (23). The enriched 14.18 scFv was subsequently inserted into pBM1.1 to fuse it with the *Pseudomonas* exotoxin A deletion mutant ETA'. In the present study we

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demonstrate the specific *in vitro* activity of this first recombinant anti-GD2 immunotoxin developed for possible clinical use in neuroblastoma.

Materials and methods

Cell lines. The human neuroblastoma cell line IMR5 obtained from Dr Unwicker (Institution of Cell Biology and Neuroanatomy, University of Marburg, Germany); the human/mouse chimeric anti-GD2 hybridoma cell line provided by Dr Reisfeld (Scripps Clinic, La Jolla, USA) and the GD2-negative, Hodgkin-derived cell line L540Cy (24) were cultivated in RPMI 1640 (Gibco, MD, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) (Gibco), 50 µg/ml penicillin (Gibco), 100 µg/ml streptomycin and 2 mM L-glutamine (Gibco). All cells were cultured at 37°C in a 5% CO₂ atmosphere. Growth medium was changed every 3 days, and cells were subcultured twice weekly.

Bacterial strains and plasmids. *E. coli* TG1 [K12 Δ(*lac-pro*), *supE*, *thi*, *hsdD5/F'traD36*, *proA⁺B⁺*, *lacI^q*, *lacZΔM15*] was used to generate filamentous phages, *E. coli* XL1-blue (*supE44*, *hsdR17*, *recA1*, *endA1*, *gyr*, *A46*, *thi*, *relA1*, *lacF'*[*pro AB⁺*, *lacI^q*, *lacZΔM15*, Tn10(*tet^r*)] for propagation of plasmids, and *E. coli* BL21(DE3) (*F⁻*, *ompT*, *hsSB(rB-MB)*, *gal*, *dcm*, *DE3*) as host for synthesis of recombinant immunotoxins. The phagemid vector pCANTAB6' (23) is derived from pHEN1 (25), carrying an additional His₆-tag for immobilized metal-ion affinity chromatography (IMAC) purification, a myc-tag for detection by anti-myc-tag antibody 9E10 (26) and a new multiple cloning site. pCANTAB6' is used for N-terminal fusion of *SfiI/NotI*-scFv fragments to the minor coat protein p3 of filamentous phage M13. The plasmid pBM1.1 (27) derived from the pET27b vector (Novagen) is used for N-terminal fusion of scFvs to a modified deletion mutant of *Pseudomonas aeruginosa* exotoxin A (28), detectable by anti-ETA monoclonal antibody (mAb) TC-1 (kindly provided by Dr Galloway, Ohio, USA). Plasmids were prepared by the alkaline lysis method and purified using plasmid kits from Qiagen (Hilden, Germany). Restriction fragments or PCR products were separated by horizontal agarose gel electrophoresis and extracted with QIAquick (Qiagen). Cloning into plasmid vectors was performed by standard methods.

Membrane preparations of tumor cells. Membranes of tumor cells were prepared as described recently (23). Briefly, IMR5 or L540Cy cells (1x10⁷ each) were suspended in PBS and washed three times. After centrifugation, sedimented cells were suspended in ice-cold 25 mM Tris-HCl buffer, pH 7.4, containing 320 mM sucrose. Membrane fractions were prepared by sequential, low-frequency sonication (Sonoplus HD 2070, Berlin, Germany). After centrifugation, the supernatant was recovered and again centrifuged. The resulting cell membrane (CM) pellet was suspended in 50 mM Tris-HCl buffer, pH 7.4, and finally centrifuged to remove loose-bound proteins from the membrane surface. The supernatant was discarded, the CM pellet suspended in 0.02 M bicarbonate buffer, pH 9.6, and stored in aliquots at -80°C until analysis. The protein concentrations of individual

membrane preparations were determined spectrophotometrically using the DC Protein Assay Kit (Bio-Rad Laboratories, Munich, Germany) with bovine serum albumin as standard. Typical yields ranged between 0.8-1.0 mg protein/ml.

Competitive phage ELISA. Cell membrane ELISA was performed as previously described (23) using ELISA microplates coated with freshly prepared membrane fractions of IMR5 cells and L540Cy cells as control and stored (until 10 months) at -80°C. 96-well Maxisorp-Plates (Nunc, Roskilde, Denmark) were coated with IMR5 cell membranes, washed 5X with TPBS and blocked with 2% (w/v) bovine serum albumin (Sigma, Deisenhofen, Germany) in PBS. After overnight incubation at 4°C, binding of the selected library (polyclonal phage ELISA) and randomly picked singular phages (monoclonal phage ELISA) was detected using peroxidase-conjugated anti-M13 antibodies (Amersham) after addition of 2',2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS[®]) solution (Boehringer Mannheim, Germany) by measuring the extinction at 405 nm with an ELISA-Reader (MWG Biotech, München, Germany). Competition experiments were performed in the presence or absence of 10 µg/ml chimeric 14.18 mAb as competitor for 14.18 scFv phages.

Construction and purification of the recombinant anti-GD2 immunotoxin 14.18(scFv)-ETA'. 14.18 scFv gene was released from phagemid vector pCANTAB6' by *SfiI/NotI* digestion and inserted into *SfiI/NotI* digested expression vector pBM1.1 containing a modified *Pseudomonas* exotoxin A gene (27). The resulting pBM1.1-14.18(scFv)-ETA' plasmid was transformed into *E. coli* BL21(DE3) (Novagen). The recombinant IT was expressed under the control of the isopropyl β-thiogalactoside (IPTG)-inducible tac promoter in the *E. coli* strain BL21(DE3) (29) as described recently (30). Briefly, bacteria were grown overnight at 26°C in Terrific Broth (TB) containing 0.5 mM ZnCl₂ and 50 µg/ml kanamycin. The culture was diluted 30-fold in 200 ml of the same medium, at an OD₆₀₀ of 2.0 supplemented with 0.5 mM sorbitol, 4% (w/v) NaCl and 10 mM betain and incubated at 26°C for additional 30-60 min. Thereafter, immunotoxin production was induced by addition of 2 mM IPTG at 26°C. Cells were harvested 15 h later by centrifugation at 3,700 g for 10 min at 4°C, then frozen at -196°C. After thawing, the cells were resuspended in 75 mM Tris/HCl pH 8.0, 300 mM NaCl, 1 capsule of protease inhibitors/50 ml (Complete[™]), Roche Diagnostics, Mannheim, Germany), 5 mM DTT, 10 mM EDTA, 10% (v/v) glycerol and sonicated 6 times for 30 sec at 200 W. The periplasmic fraction was recovered as supernatant after centrifugation at 21,000 g_{av} for 30 min at 4°C and transferred to 75 mM Tris/HCl, pH 8.0, 1 M NaCl and 10% (v/v) glycerol using Hitrap desalting columns (Pharmacia, Germany, Freiburg). Recombinant immunotoxin was partially purified by immobilized metal-ion affinity chromatography (IMAC) using nickel-nitriloacetic acid (Ni²⁺-NTA) chelating sepharose (Qiagen) on a BioLogic workstation (Bio-Rad, München, Germany). Bound protein was eluted with 250 mM imidazole in 75 mM Tris/HCl, pH 8.0, 1 M NaCl, 10% (v/v) glycerol. Fractions containing 14.18(scFv)-ETA' were pooled

and concentrated by ultrafiltration. Functional recombinant immunotoxin was finally purified using size exclusion chromatography (SEC) using Bio-Prep SE-100/17 (Bio-Rad) on the BioLogic workstation by separation in PBS, pH 7.4. Purified protein was analyzed by SDS-PAGE and quantified by densitometry (GS-700 Imaging Densitometer, Bio-Rad) after Coomassie staining in comparison with BSA standards.

Binding analyses. The binding activity of 14.18(scFv)-ETA' was determined on membrane preparations of IMR5 cells by cell membrane ELISA. Samples were incubated with anti-ETA' mAb TC-1 for 1 h. After washing, 100 μ l of (Fab'2) fragments of peroxidase (POD) coupled goat anti-mouse-IgG (Roche Diagnostics, Mannheim, Germany) (1:5,000 in TBS containing 0.5% (w/v) BSA and 0.05% (v/v) Tween 20) were added and samples incubated for 1 h at room temperature. Bound 14.18(scFv)-ETA' was detected by addition of 100 μ l ABTS® solution (Boehringer) by measuring the extinction at 405 nm with an ELISA-Reader (MWG Biotech).

Cell binding activity was also evaluated by flow cytometry. Bound immunotoxin was documented using mAb TC-1 and fluorescein isothiocyanate (FITC) labeled goat anti-mouse immunoglobulin on a FACScan (Becton Dickinson, Heidelberg, Germany).

Colorimetric cell viability assay. Metabolism of the yellow tetrazolium salt, XTT, to a water soluble orange formazan dye was determined according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). Various dilutions of 14.18(scFv)-ETA' were distributed in 100 μ l aliquots in 96-well plates. $2-4 \times 10^4$ target cells in 100 μ l aliquots of complete medium were added and the plates incubated for 48 h at 37°C. Subsequently, the cell cultures were pulsed with 100 μ l fresh culture medium supplemented with XTT and N-methyl dibenzopyrazine methyl sulfate (PMS) (in final concentrations of 1.49 mM and 0.025 mM respectively) for 12 h. The spectrophotometrical absorbance of the samples was measured at 450 and 650 nm (reference wavelength) with an ELISA reader (MWG Biotech). The concentration required to achieve 50% reduction of cell viability relative to untreated control cultures (IC_{50}) was calculated as previously described (30). All measurements were done in triplicate.

Results

Cloning of V-genes and selection of the 14.18 scFv. After extraction of total RNA from ch14.18 cells, the variable domains of heavy (VH) and light (VL) chain immunoglobulin genes were amplified by means of RT-PCR using an improved set of oligonucleotides and assembled by Splice Overlap Extension (SOE)-PCR (31) with a (Gly₄Ser)₃ linker (23). The resulting single chain variable fragments (scFv) were cloned into phagmid vector pCANTAB6' for expression as fusion proteins with the bacteriophage coat protein p3 or as a soluble scFv fragment, depending on the expression conditions (32).

Binding properties of 14.18 scFv phages. Specific binding of 14.18 scFv phages to IMR5 cells was demonstrated by means

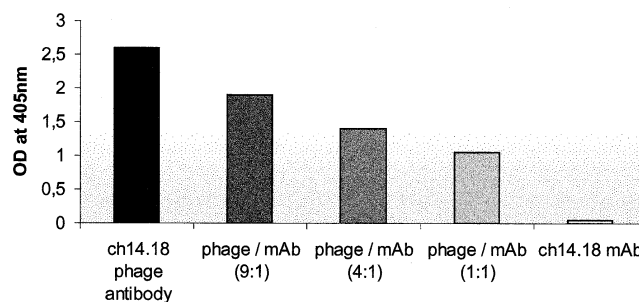


Figure 1. Competitive ch14.18 phage ELISA on IMR5 membranes. Different dilutions of ch14.18 hybridoma supernatant (10 μ g/ml) and 14.18 phage antibodies 1.3×10^{10} were used for competition. Binding of phages was detected with peroxidase-conjugated anti-M13 monoclonal antibody using ABTS solution as substrate.

of cell membrane ELISA (Fig. 1). To verify binding specificity of the selected 14.18 scFv phage to the GD2 epitope recognized by the monoclonal ch14.18 antibody, competition experiments were performed. A reduced staining is visible if 14.18 scFv displayed on phage was simultaneously incubated with increasing amounts of the parental ch14.18 mAb. A control experiment with phage displaying no scFv on their surface exhibits only background staining of the IMR5 cells.

Construction and purification of the recombinant 14.18(scFv)-ETA' immunotoxin. After subcloning of the 14.18 scFv gene into the bacterial expression vector pBM1.1 and transformation of *E. coli* BL21(DE3), bacteria were grown under osmotic stress conditions in the presence of betaine. The 70 kDa 14.18(scFv)-ETA' was functionally accumulated in the periplasmic space and subsequently purified by combinations of IMAC and size exclusion chromatography (Fig. 2). The recombinant immunotoxin was substantially stabilized during purification by 1 M NaCl, eluted from Ni²⁺-NTA columns by 250 mM imidazole and separated by size exclusion chromatography between 20 and 100 kDa. Only intact 14.18(scFv)-ETA', but no degradation products nor rIT aggregates were collected.

Specific binding and cytotoxicity of 14.18(scFv)-ETA'. Binding activity of purified 14.18(scFv)-ETA' towards the GD2-positive target cell line IMR5 and the GD2-negative cell line L540Cy was investigated by flow cytometry (Fig. 3) and ELISA. 14.18(scFv)-ETA' bound to IMR5 but not to L540Cy cells. Specific cytotoxicity of 14.18(scFv)-ETA' was demonstrated in a cell viability assay (Fig. 4). 14.18(scFv)-ETA' developed cytotoxic activity against the GD2-positive cell line IMR5 ($IC_{50} = 0.326 \mu$ g/ml). There was no unspecific toxicity against the GD2 negative Hodgkin-derived cell line L540Cy.

Discussion

In this study, we report construction and functional properties of the first recombinant immunotoxin directed against the disialoganglioside GD2 for possible clinical use in patients with neuroblastoma. The major findings to emerge from

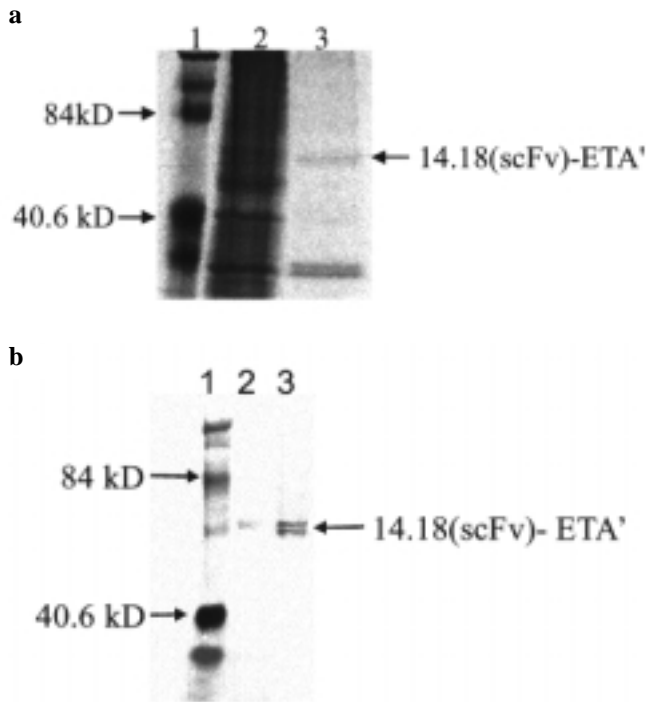


Figure 2. Purification of 14.18(scFv)-ETA' after metal chelate affinity chromatography. (a), Protein was electrophoresed through a 10% polyacrylamide gel and visualized with Coomassie Blue (lane 1, molecular weight marker; lane 2, whole cell extract; lane 3, eluate of Ni-NTA resin). (b), Western blotting was performed, using anti-ETA monoclonal antibody TC-1 (lane identification is the same as panel 2a).

our study are: i) 14.18-scFv phage antibody selected by phage display on the neuroblastoma cell line IMR5 showed GD2-specific binding activity as documented by competition experiments; ii) 14.18(scFv)-ETA' isolated from the periplasmic space of *E. coli* cultured under osmotic stress conditions in the presence of compatible solutes was purified by a combination of immobilized metal affinity and molecular size chromatography; iii) 14.18(scFv)-ETA' binds to the GD2⁺ cell line IMR5 as documented by CM-ELISA and flow cytometry; iv) the 14.18(scFv)-ETA' immunotoxin showed specific toxicity against the GD2⁺ neuroblastoma cell line IMR5 (IC₅₀ = 0.326 µg/ml).

Gangliosides are mediators for intracellular signal transduction (33) and intercellular communication (34). Altered ganglioside biosynthesis during malignant transformation results in expression of a qualitatively and quantitatively different ganglioside profile in many tumors of neuroectodermal origin (35). The diversity of sugar residues creates a variety of epitopes which can be recognized by different antibodies. Aberrant ganglioside expression by tumor cells, e.g. GD2, GD3 and GM2, have been proven to be suitable targets for immunotherapy with monoclonal antibodies (36) and immunotoxins (14).

The current major obstacles in cancer therapy include acquisition of multidrug resistance (37) resulting in residual tumor cells surviving first line treatment. These cells often have a 'dormant' state, since many rest in G₀ phase (38). Toxins and conventional chemotherapeutic agents have different mechanisms of action. Therefore, cancer cells that

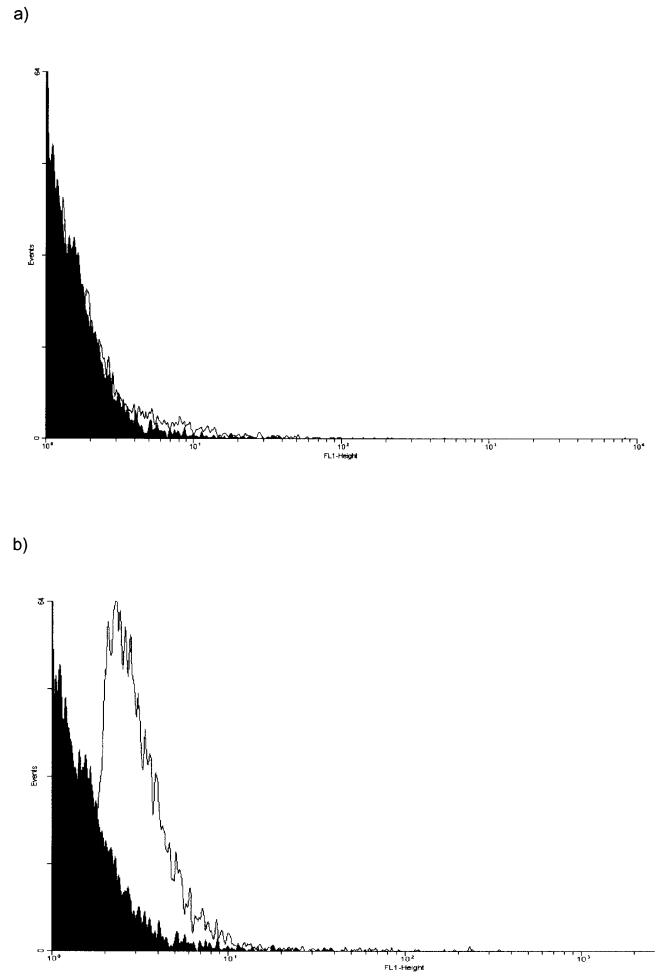


Figure 3. Binding activity of 14.18(scFv)-ETA' to GD2⁺ cell line IMR5. Evaluation by flow cytometry. Binding of 14.18(scFv)-ETA' to GD2-negative cell line L540Cy (a) and GD2-positive neuroblastoma-derived cell line IMR5 (b). Cells were incubated with 14.18(scFv)-ETA', anti-ETA mouse IgG (TC-1) and FITC-conjugated anti-mouse IgG (white curves) or as negative controls with PBS instead of 14.18(scFv)-ETA' (black curves). Histograms represent logarithms of FITC fluorescence as documented using a FACScan.

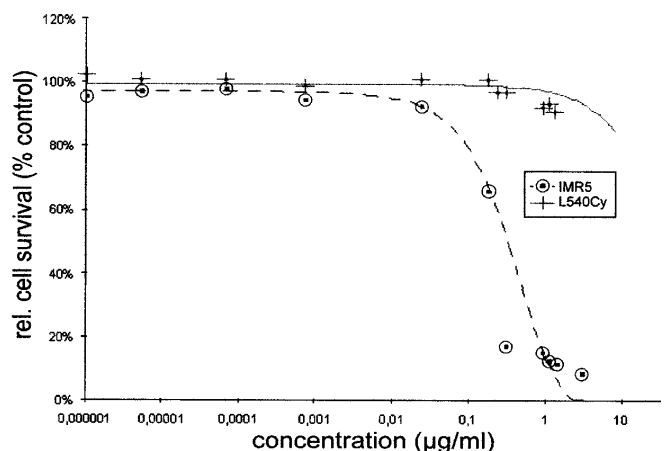


Figure 4. Growth inhibition of Hodgkin-derived cell lines after incubation with 14.18(scFv)-ETA' as documented by a cell viability assay. IMR5 (GD2⁺) or L540Cy (GD2⁻) were treated with dilutions of the immunotoxin and their ability to metabolize the tetrazolium salt, XTT, to a water soluble formazan salt (formed by mitochondrial dehydrogenase activity) was measured as absorbance at 450 and 650 nm (reference wavelength).

are naturally resistant or acquire resistance to chemotherapeutic agents might not be cross-resistant to toxin-based therapies. Furthermore, toxins are potentially cytotoxic for non-dividing cells that cannot be killed by conventional chemotherapy. These unique properties make them attractive for use in the treatment of cancer. Thus, peptidic toxins such as *Pseudomonas* exotoxin, *Diphtheria* toxin and Ricin have been chemically or genetically attached to monoclonal antibodies (39,40) in order to create specific anti-cancer drugs which can overcome drug resistance and kill dormant cells. Several mAbs against antigens on neuroblastoma cells were chemically-linked to ricin A-chain and evaluated for their *in vitro* and *in vivo* antitumor activity (14). The most potent immunotoxin, 14G2a.dgA, very efficiently inhibited protein synthesis of IMR5 cells ($IC_{50} = 6 \times 10^{-12}$ M). Since recombinant DNA technology allows the construction and possible modification of immunotoxins with relative ease, we developed a new anti-GD2 rIT based on a *Pseudomonas* exotoxin derivative (ETA'). For this purpose, we isolated the V-genes from this antibody from the available hybridoma ch14.18, a humanized switch variant of the original antibody. After assembly and cloning into phagemid pCANTAB6', we recently developed a new protocol for the selection of scFv phages on intact cells to significantly reduce the amount of insert-free phages (23). We isolated 14.18(scFv)-ETA' from periplasmic space with a substantial proportion of biologically active protein. The *in vitro* potency of the new recombinant anti-GD2 immunotoxin 14.18(scFv)-ETA' described in the present study is dramatically reduced compared to the chemically-linked immunotoxin (14). This loss of toxicity might be due to reduced affinity or avidity of the scFv compared to the maternal monoclonal antibody and might be improved by affinity maturation or 'rational' engineering (41-43).

In summary, the newly constructed recombinant anti-GD2 immunotoxin 14.18(scFv)-ETA' is effective and specific against GD2⁺ cell lines and might therefore be a good candidate for clinical evaluation in patients with neuroblastoma.

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