A Recombinant CD7-specific Single-Chain Immunotoxin Is a Potent Inducer of Apoptosis in Acute Leukemic T Cells

Matthias Peipp, Heide Küpers, Domenica Saul, Beate Schlierf, Johann Greil, Susan J. Zunino, Martin Gramatzki, and Georg H. Fey

Chair of Genetics [M. P., H. K., D. S., S. J. Z., G. H. F.], Institute of Biochemistry [B. S.], Division of Hematology/Oncology, Department of Medicine III [M. G.], University of Erlangen-Nuremberg, D-91054 Erlangen, Germany, and Department of Pediatric Oncology, Children’s Hospital, University of Tübingen, D-72076 Tübingen, Germany [J. G.]

ABSTRACT

A recombinant immunotoxin was constructed from the hybridoma antibody TH-69 directed against human CD7, a surface antigen of leukemic T cells. The antibody was subcloned as a single chain Fv (scFv) fragment and genetically linked to a truncated Pseudomonas exotoxin A fragment containing the catalytic domains II and III but lacking the receptor binding domain I. Domain I was replaced by the scFv, thus conferring restricted specificity for CD7-positive cells. The bacterially expressed and purified toxin retained binding specificity for CD7-positive cells. It promoted apoptosis in two CD7-positive cell lines derived from T-lineage acute lymphoblastic leukemias, CEM and Jurkat, but not in the CD7-negative B-lymphoid lines REH, Nalm-6, and SEM. Maximum killing in excess of 95% was reached after 96 h in CEM and Jurkat cells with a single dose of 100 ng/ml. Cells treated with a similarly constructed scFv-exotoxin A immunotoxin against melanoma-associated chondroitin sulfate proteoglycan, an antigen absent from leukemic T cells, remained unaffected. Lysis of target cells occurred via apoptosis as evidenced by staining with Annexin V and specific cleavage of poly(ADP-ribose) polymerase. Approximately 20% of leukemic cells from a patient with CD7-positive acute T-cell leukemia kept in long-term primary culture for 30 cell generations were killed within 96 h after treatment with the toxin. These findings justify further evaluation of the agent in view of potential therapeutic applications.

INTRODUCTION

Antibodies have attracted renewed strong interest as therapeutics in clinical oncology. The CD20 antibody rituximab (1), the anti-Her2/neu breast cancer drug Herceptin (2), and the CD33 immunomucoglycan antibody, alemtuzumab (3) all have impressive activity in tumor patients. However, patients suffering from T-cell leukemias and lymphomas still have very limited treatment options. The 5-year survival for most of these patients is <30%, and this category ranks as the worst in lymphoma prognosis (4). The CD52 antibody CAMPATH-1H (Alemtuzumab), recently approved by the Food and Drug Administration for the treatment of B-cell chronic lymphocytic leukemia, is also effective against certain T-cell neoplasias (5). However, this antibody leads to a prolonged and almost complete depletion of T and B lymphocytes, with a considerable frequency of opportunistic infections. Prognosis of childhood T-ALL (6) has improved with modern chemotherapy, but T-ALL patients with remission induction failure after induction chemotherapy or with relapse of T-ALL still have a very poor prognosis (6).

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3 To whom requests for reprints should be addressed, at Chair of Genetics, University of Erlangen Nuremberg, Staudtstrasse 5, D-91058 Erlangen, Germany. Phone: 49-9131-852-8493; Fax: 49-9131-852-8526; E-mail: gfey@biologie.uni-erlangen.de.

4 M. Gramatzki, unpublished data.
specific antibody, showed clinical efficacy in Phase I trials (30, 31). The use of this toxin, however, was limited by its instability and associated vascular toxicity. Therefore, current efforts are directed at linking the ricin A toxin by recombinant DNA procedures to an scFv fragment of a CD7-specific antibody, but results of this effort are still unknown (27). The toxin saporin was linked via a bifunctional cross-linker and a disulfide bridge (32), and pokeweed antiviral protein was chemically linked to CD7-specific antibodies (28). In previous attempts, our group has linked cis-platinum to the TH-69 antibody, and this combination caused an inhibition of proliferation and a direct cytotoxic effect in the T-cell line CEM (33). Therefore, CD7 meets the requirements for a potentially useful target for immunotoxins. However, until now, most CD7-directed immunotoxins were generated by conventional chemical coupling and suffered from instability and accompanying side effects, such as vascular toxicity.

To overcome some of these shortcomings, two key steps were taken in the present study. One was the generation of a stable peptide bond between the scFv antibody fragment and the toxin by recombinant genetics. The other was the use of a truncated toxin, which was unable to enter human cells on its own, in case it should be cleaved from the antibody moiety. This truncated toxin was devoid of its own binding domain for receptors on mammalian cells and, therefore, was expected to generate significantly fewer side effects. The recombinant toxin created here can only be absorbed by CD7-positive cells by virtue of its scFv portion and the CD7 internalization mechanisms of these cells.

The toxin used in our approach was Pseudomonas ETA3, specifically the truncated version lacking domain I and containing only domains II and III (34). Domain I is the binding domain for a cell surface receptor present on most mammalian cells. The intact toxin enters mammalian cells via binding of domain I to this receptor. Domains II and III are required for intracellular transport and carry the active center of the toxin, respectively, which causes apoptosis by inhibiting protein synthesis via a block of the translation elongation factor-2 (35). Consequently, the truncated version of ETA lacking domain I is not toxic as long as it remains in the extracellular space. In addition, the truncated form of Pseudomonas ETA is expected to have fewer side effects on vascular endothelial cells, because it has an ~1000-fold lower relative affinity to the LDV receptor on human vascular endothelial cells than, e.g., ricin A (36). Once coupled to an antibody directed against an antigen capable of internalizing, the truncated ETA becomes a potent immunotoxin, e.g., CD22- and CD25-specific scFvs linked to truncated ETA were remarkably efficient immunotoxins for the treatment of hairy cell leukemias and CD25-specific scFvs linked to truncated ETA becomes a potent immunotoxin, the antibody directed against an antigen capable of internalizing, the T-cell line CEM (33). Therefore, CD7 meets the requirements for a potentially useful target for immunotoxins. However, until now, most CD7-directed immunotoxins were generated by conventional chemical coupling and suffered from instability and accompanying side effects, such as vascular toxicity.

Materials and Methods

Bacterial Strains and Plasmids. Escherichia coli XL1-Blue (Stratagene, Amsterdam, the Netherlands) was used for the amplification of plasmids and cloning and E. coli TG1 (from Dr. G. Winter, MRC, Cambridge, United Kingdom) for the screening of antibody libraries. Libraries were generated in the phagemid vector pAK100, and pAK400 was used for the expression of soluble scFvs (39). E. coli BL21 (DE3; Novagen, Inc., Madison, WI) was used for the expression of scFv-ETA fusion proteins.

Culture of Eukaryotic Cells. Leukemia-derived SEM cells (40), CEM cells (41), Jurkat cells (42), REH cells (DSMZ, German Collection of Microorganisms and Cell Lines, Braunschweig, Germany), Nalm-6 cells (DSMZ), MV4;11 cells (American Type Culture Collection, Rockville, MD), and the two hybridomas TH-69 (26) and 9.2.27 (anti-MCSP; Ref. 43) were cultured in RPMI 1640-Glutamax-I (Invitrogen, Karlsruhe, Germany) containing 10% FCS and penicillin and streptomycin at 100 units/ml and 100 μg/ml, respectively. COS monkey cells (DSMZ) were maintained in DMEM-Glutamax-I medium containing 10% FCS, penicillin, and streptomycin. The LLC cell line was established from bone marrow lymphoblasts of a 6-month-old female infant diagnosed with ALL.1 LLC cells (44) were cultured in Iscove’s Modified Dulbecco Medium (Life Technologies, Inc.), supplemented with human transferrin and linoleic acid at 3 μg/ml, BSA at 10 mg/ml, α-thioglycolate at 50 mg/ml, bathocuproin-disulfonic acid at 20 μM, insulin at 1 μg/ml, and recombinant human interleukin-7 (Strathamann, Inc., Hamburg, Germany) at 10 ng/ml.

Patient-Derived Leukemic Cells. HCL cells were derived from a 10-year-old patient, diagnosed with precursor T-ALL (CD1a+, CD2−, CD4−, CD7+, CD8−, and CD3+) at the Children’s Hospital of the University of Erlangen. He was treated according to ALL-BFM-86 protocol and reached remission at day 33 of protocol I. The culture was established from peripheral blood, and the cells proliferated with a doubling time of 3 days. They were cultured in the same media as described for the cultivation of LLC cells. After 60 generations, the cells entered a proliferative crisis, and the majority of the cells died. Sufficient aliquots were frozen from early passages (generation 30) to allow reproducible work with cells of a defined number of generations in culture. The cultured cells continued to express high levels of CD7. In addition, fresh cells from a 27-year-old male patient with pre-T-ALL were tested. The immunophenotype of bone marrow blasts (80% marrow infiltration) initially was CD7+/CD5+/cytoplasmic CD3+/CD34+/CD11c−/CD3−. Cells showed Terminal deoxynucleotidyl transferase positivity. Tests were performed after the patient was referred to our university hospital after initially being treated elsewhere with one course of cyclophosphamide-Adriamycin-vincristine-prednisone chemotherapy for aggressive lymphoma.

Preparation of scFv Phage Display Libraries. Total RNA was prepared with Trizol (Invitrogen) from the CD7 antibody producing hybridoma, clone TH-69, and the hybridoma 9.2.27, producing a control antibody against MCSP. First-strand cDNA was prepared from 10–15 μg of total RNA (39). PCR amplification of immunoglobulin variable region cDNAs and cloning into the phagemid vector pAK100 was performed as described (39, 45). Propagation of combinatorial scFv libraries and filamentous phages was performed after published procedures (45).

Panning of Phage Display Libraries with Intact Cells. Panning of phage display libraries with intact cells was carried out using CD7-positive E. coli and COS cells positive for the MCSP antigen. For this purpose, 5 × 10⁸ cells were incubated in PBS (Invitrogen, Groningen, the Netherlands) containing 2% nonfat dry milk (NM-PBS) to block nonspecific binding sites and then incubated with 500 μl of the scFv phage library for 1.5 h at 25°C under slow agitation. Cells were washed 10 times with 2% NM-PBS and twice with PBS. Bound phages were eluted by adding 50 μg of the parental antibody as competitor. After incubation for 10 min at 25°C, cells were sedimented by centrifugation, and the supernatant was used to infect 10 ml of exponentially growing E. coli TG1 cultures. A total of 20 ml of 2 × YT medium (16 grams/liter trypotone/peptone (Roht, Karlsruhe, Germany), 10 grams/liter yeast extract (Roht), and 5 grams/liter NaCl) containing 1% glucose and chloramphenicol at 30 μg/ml was added, and the cells were incubated for 2 h at 37°C under vigorous shaking (250 rpm). The cells were then superinfected with helper phage. A total of 70 ml of 2 × YT medium was then added, and isopropylthiogalactoside was added at a final concentration of 0.5 mM. Two h after infection, kanamycin was added to a final concentration of 25 μg/ml and the culture was grown overnight at 30°C. On the next day, phages were prepared as described (45). After two rounds of panning, individual phages were purified, and the inserts were sequenced (46) using an Applied Biosystems automated DNA sequencer (ABI Prism 310 Genetic Analyzer, Perkin-Elmer, Ueberlingen, Germany).

Bacterial Expression and Purification of Soluble scFv Fragments. For the soluble expression of antibody fragments, cDNAs coding for CD7- and MCSP-specific scFvs were subcloned into the expression vector pAK400, and the plasmids were propagated in E. coli HB2151 (from Dr. G. Winter; MRC, Ueberlingen, Germany).

5 J. Greil, unpublished data.
CD7-EXOTOXIN A scFv IMMUNOTOXIN

Cambridge, United Kingdom). Expression and purification of CD7- and MCSP-specific scFvs were performed as described (45).

Construction and Expression of scFv-ETA Fusion Proteins. For periplasmic expression of scFv fragments fused to truncated ETA under the control of the inducible T7 promoter, the plasmid pet27b(+) (Novagen, Inc.) was modified as follows. To introduce a 6 × His-tag at the NH₂ terminus and a SfiI-site, two oligonucleotides, oligo 1: 5'-ccc cat cac cat cac cat cac ggg gcc cag cgc g-3' and oligo 2: 5'-ga tgc ggc cgg ctt ggc ccc gtt atg atg atg atg ggc-3' (MWG-Biotech, Munich, Germany) were hybridized and ligated into pet27b(+) digested with MscI and BamHI. In a second step, a DNA fragment coding for a 20 amino acid linker with a second SfiI site was inserted into HimHI/NorI-digested modified pet27b(+) using oligonucleotides 3 and 4 (oligo 3: 5'-agg gcc cgg gcc ggc gat ggt ggt ggt ggt gcc ggc ggc gat ggt ggt ggt ggt ggt ggt ggt ggt ggt ggc ggc ggc ggc ggc ggc gtc tcg-3' and oligo 4: 5'-gcg cgc cgc ggc gcc ggc gcc ggc gcc ggc ggc ggc gcc ggc-3'). This procedure resulted in the construct pet27b(+)-L3HS9. Sequences coding for CD7- and MCSP-specific scFvs were excised from the pAK400-anti CD7 and pAK400-anti MCSP expression constructs harboring the corresponding scFv fragments and cloned as SfiI-cassettes into pet27b(+)-L3HS9. The resulting plasmids were digested with NotI/CelI, and a DNA fragment encoding the truncated ETA was amplified by PCR from plasmid pSW202 (47) using the primers oligo 5: 5'-gat cgg cgc ggc ggc aag ggg cgg cag cgt gg-3' and oligo 6: 5'-cag tag gtc cag ggc agt ttc ttc gg-3'). The amplified DNA was then ligated into the vector and sequenced.

The scFv-ETA fusion proteins were expressed under osmotic stress as described (48). Induced cultures were harvested 16–20 h after induction. The bacterial pellet from a 1 liter culture was resuspended in 10 ml of isosf-buffer [50 mM NaH₂PO₄, 300 mM NaCl, and 10 mM imidazol (pH 8.0)]. Lysozyme was added at a final concentration of 1 mg/ml, and the suspension was incubated for 30 min on ice. Cells were disrupted three times for 1 min at 120 W in a sonicator/cell disrupter (B. Braun Biotech, Melsungen, Germany). The scFv-ETA fusion proteins were enriched by affinity chromatography using nickel-nitrilotriacetic agarose Beads (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

Flow Cytometric Analysis. The binding of scFvs to cells was analyzed using a FACSCalibur FACS instrument and CellQuest software (Becton Dickinson, Mountain View, CA). Cells were stained with scFv fragments as described (45). Ten thousand events were collected for each sample, and analyses of whole cells were performed using appropriate scatter gates to exclude cellular debris and aggregates. To monitor binding of scFv-ETA fusion proteins, 5 × 10⁶ cells were incubated for 30 min on ice with 20 μl of the immunotoxin at a concentration of 1 μg/ml. A nonrelated immunotoxin served as a control for background staining. The cells were washed with PBA buffer [containing PBS, 0.1% BSA, and 7 mM Na-azide] and then incubated with 50 μl of a polyclonal rabbit anti-Pseudomonas ETA serum (Sigma, Deisenhofen, Germany) diluted 1:250 in PBA buffer. Cells were washed and incubated with fluorescein-isothiocyanate (FITC)-conjugated pig antirabbit-IgG (DAKO Diagnostica GmbH, Hamburg, Germany) for 30 min. After a final wash, the cells were analyzed by FACS.

Measurement of Cytotoxic Effects of Immunotoxins. Cells were seeded at 2.5 × 10⁵/ml in 24-well plates, and immunotoxins were added at varying concentrations. Cell death was measured by staining nuclei with a hypotonic solution of PI as described (49, 50). The extent of cell death was determined by measuring the fraction of nuclei with subdiploid DNA content. Fifteen thousand events were collected for each sample and analyzed for subdiploid nuclear DNA content. To determine whether cell death was attributable to apoptosis, whole cells were stained with FITC-conjugated Annexin V (PharMingen, Heidelberg, Germany; Ref. 51) and PI in PBS according to the manufacturer’s protocol. For blocking experiments, cells were seeded at 2.5 × 10⁵/ml in 24-well plates, and a 100-fold molar excess of the parental antibody or a nonrelated antibody of the same isotype was added to the culture 1 h before adding the immunotoxin (100 ng/ml). Viable cell counts were determined by trypan blue staining. Cell death was quantitated by staining with Annexin V and PI in PBS as described above.

SDS-PAGE and Western Blot Analysis. SDS-PAGE was performed by standard procedures (52). Western blots were performed with secondary antibodies coupled to horseradish peroxidase (Dianova, Hamburg, Germany; Ref. 53). Enhanced chemiluminescence reagents (Amersham Pharmaccia, Freiburg, Germany) were used for detection. ScFvs were detected with a penta-His antibody (Qiagen). ScFv-ETA fusion proteins were detected using an anti-Pseudomonas ETA polyclonal antibody (Sigma). Full-length PARP and its specific cleavage product were detected using a mouse antihuman PARP antibody (PharMingen).

Statistical Analysis. All statistical analyses were performed with Microsoft EXCEL software. P values were obtained using two-tailed paired t tests with a confidence interval of 95% for evaluation of the significance of differences between treatment groups.

RESULTS

The Recombinant CD7-specific scFv Fragment and the Derived Immunotoxin Retain Specific Binding. Two scFv fragments, one directed against CD7 and the other specific for the MCSP antigen, were subcloned from the corresponding hybridoma cell lines. For this purpose, the light and the heavy chain variable regions were PCR amplified and connected via overlap-extension PCR. The scFv fragments were then ligated into the phagemid vector pAK100. Phage display libraries in this manner were used to identify specific binders by screening on antigen-positive cells for two consecutive rounds. After the second round of panning, individual phages were analyzed by cellular ELISA. ScFvs from phages with the strongest binding to antigen-positive cells were subcloned into the expression vector pAK400, a vector designed for the expression of soluble scFv fragments. Purified scFv fragments from periplasmic extracts were analyzed for binding by FACS. The recombinant CD7-specific scFv fragment subcloned in this manner from the TH-69 hybridoma retained specific binding for the CD7-positive T-cell line CEM (Fig. 1A) but failed to bind to the CD7-negative pro-B leukemia cell line SEM (Fig. 1B). The MCSP-specific control scFv subcloned from the hybridoma cell line 9.2.27 specifically bound to MCSP-positive COS cells (Fig. 1C) but failed to bind to the MCSP-negative cell line MV4–11 (Fig. 1D). Thus, the process of subcloning the hybridoma antibodies to scFv fragments did not detectably alter the binding specificity of the antigen-combining sites.

A scheme for the construction of the recombinant immunotoxin is given in Fig. 2. The protein was purified by affinity chromatography to >90% purity, and ~100 μg of purified protein were routinely

Fig. 1. The subcloned scFv fragments against CD7 and MCSP specifically bind to antigen-positive cells. Cells were stained with purified scFv fragments (black) or with a nonrelated scFv (white) at the same concentration and analyzed by FACS. A, CD7-positive CEM cells stained with CD7-specific scFvs. B, CD7-negative SEM cells stained with CD7-specific scFvs. C, MCSP-positive COS cells stained with anti-MCSP scFvs. D, MCSP-negative MV4–11 cells stained with anti-MCSP scFvs. The data are representative of four separate experiments.
obtained from 1 liter of bacterial culture. The protein apparently retained its native folding after expression in bacteria, because it was not necessary to denature and renature it to obtain efficient specific binding after purification. The protein also did not show a noticeable tendency to form intracellular precipitates. Coupling of the scFv coding sequences to the truncated ETA coding sequences via sequences coding for a polypeptide linker also did not affect the binding specificity of the antigen-combining site. The recombinant CD7-specific toxin still bound to CEM (Fig. 3A) but not to CD7-negative SEM cells (Fig. 3B). An additional control was performed to exclude that any recombinant scFv-ETA protein may have these binding properties because of their common design rather than their particular scFv-moiety. To this effect, the hybridoma 9.2.27-derived antibody specific for the MCSP antigen (43) was also subcloned into an scFv fragment and converted into an anti-MCSP-ETA scFv immunotoxin by analogous procedures. This molecule was similarly expressed in bacteria and purified. It failed to bind to MCSP-negative CEM cells (Fig. 3D) but to LLM cells, a leukemia-derived pro-B cell line with chromosomal translocation t(4;11) (Fig. 3C) that expresses MCSP on the cell surface. Thus, the specificity of binding of the scFv-ETA immunotoxins was the result of their particular scFv moieties and not the result of their common design.

The Recombinant CD7-ETA Immunotoxin Promotes Specific Death of Antigen-positive Cells at Nanomolar Doses. To investigate the cytotoxic properties of the recombinant CD7-specific immunotoxin, the agent was added to CEM and SEM cells, and viable cells were counted by using trypan blue exclusion. Cell death was antigen specific, because it was blocked by pretreatment with excess concentrations of the parental monoclonal CD7 antibody, TH-69, but not with similar concentrations of an isotype control antibody (Fig. 4). Next, the extent of cell death was measured as a function of time and the dose of the agent by staining of nuclei with PI and FACS. The fraction of cells with subdiploid DNA content was equated with the fraction of dead cells. The immunotoxin was added to the cultures in a single dose, and culture aliquots were analyzed for cell death at different times after addition. The toxin promoted death of CEM cells in a time- and dose-dependent manner (Fig. 5A) but failed to affect SEM cells at the same concentrations (Fig. 5B). Significant cell death was already obtained at concentrations of 1 ng/ml, compared with untreated cells (P = 0.002), and maximum death was reached at ~100 ng/ml, corresponding to a concentration of 1.3 nM. Thus, the agent was capable of killing CD7 antigen-positive cells with high efficiency within 96 h after the addition of a single dose in the nanomolar concentration range. The immunotoxin also killed cells from a second leukemia-derived T-ALL line, Jurkat, at similar concentrations. At concentrations causing maximum death of Jurkat cells, the B-leukemic cell lines REH, Nalm-6 (Fig. 6), and SEM remained unaffected (Fig. 5B). Thus, it can be excluded that the toxin killed nonspecifically, and the data support the conclusion that the cytotoxic effect was antigen-specific. The fraction of dead cells measured by this procedure did not reach 100%, although microscopic analysis suggested near complete lysis. This apparent discrepancy was resolved and found to be the result of this particular method of quantitating cell death. When a different method was used, the fraction of dead cells exceeded 95% under identical conditions (see below).

Cell Death Induced by the CD7-ETA Immunotoxin Occurs via Apoptosis. To investigate whether the observed cell death was attributable to apoptosis, CEM and Jurkat cells were treated with CD7-ETA or with the control toxin and then stained with Annexin V and PI. After the exposure (48 h), 30% of CEM cells and 24% of Jurkat cells stained positive for Annexin V and negative for PI, a characteristic sign of death by apoptosis (Fig. 7A, bottom right quadrant). As a control, the recombinant anti-MCSP-ETA scFv immunotoxin of similar design failed to induce Annexin V staining and cell death. As an additional sign of death by apoptosis, the CD7-ETA toxin induced cleavage of PARP from its intact form (116 kDa) to its characteristic 85 kDa proteolytic fragment in CEM cells. Treatment of these cells with equivalent concentrations of the anti-MCSP-ETA toxin failed to...
induce PARP cleavage (Fig. 7B). Thus, by two independent criteria, the CD7-specific immunotoxin was shown to induce cell death via apoptosis for both the CEM and Jurkat lines (Fig. 7, A and B).

A direct comparison of the two methods used to quantitate cell death was performed. After (96 h) the addition of the toxin at 100 ng/ml, cell death was measured either by staining with PI in hypotonic solution (method 1; Fig. 8A) or by staining with PI (PI uptake) and Annexin V (method 2; Fig. 8B). Method 1 showed ~80% of dead cells (subdiploid DNA content), whereas measurement by method 2 showed a significantly higher number of dead cells (P = 0.04). Thus, measurement by method 1 underestimated the number of dead cells relative to determination by microscopic counting and method 2 (Fig. 8, A and B). Apoptotic death induced by the toxin and evidenced by method 2 was inhibited specifically by pretreatment with an excess of the parental monoclonal TH-69 antibody (Fig. 9, middle two panels). Treatment with a comparable excess of an isotype control antibody (Fig. 9, bottom two panels) showed cell death to the same extent as cells treated with the CD7-ETA toxin alone (Fig. 9, top two panels). These results confirm the conclusion that killing was antigen specific and that induction of apoptosis was attributable to receptor-mediated uptake of the immunotoxin.

The Recombinant CD7-ETA Immunotoxin Promotes Specific Lysis of Short-term, Cultured Leukemic Cells. To be of therapeutic interest, the toxin needs to lyse not only stable leukemia-derived...
For this purpose, a low-passage culture of cells derived from a pediatric patient (HOL) with T-ALL was used. HOL cells had been kept in culture for 30 generations. Their generation time was 3 days as opposed to 22–24 h for the cell lines CEM and Jurkat. Treatment of CD7-positive HOL cells with the immunotoxin also caused apoptosis, although less efficient than the effect seen after treatment of the stable lines (Fig. 10). Compared with the MCSP-ETA-treated controls, 20% specific death (Annexin V positive; \( P = 0.007 \)) was observed 96 h after administration of an initial dose of 100 ng/ml and a second dose after 48 h, given with a renewal of the culture medium. The effect was specific, because it was prevented by pretreatment with an excess of the parental TH-69 antibody but not by pretreatment with a comparable excess of a control antibody of matching isotype (\( P = 0.03 \)). Pretreatment with the parental antibody did not completely block CD7-ETA-induced killing, but the difference in cell death at time point 96 h between MCSP-ETA and CD7-ETA blocked by TH-69 did not reach statistical significance (\( P = 0.08 \)). The anti-MCSP-ETA scFv immunotoxin had no effect on HOL cells, which are MCSP negative. In conclusion, the CD7-ETA immunotoxin was able to induce cell death in short-term cultures of primary leukemic cells but not to the same extent as in established cell lines, although the toxin was added twice.

Similar results were obtained by analyzing freshly drawn blood cells from an adult patient treated for pre-T-ALL. Immunophenotyping revealed 23% blasts before exposure to the CD7-ETA immunotoxin. The agent was added in a single dose of 100 ng/ml, and cell death was measured by FACS analysis using Annexin V staining and PI uptake. After 24 h, 12% specific cell death was observed and 20% after 48 h. Fresh peripheral blood mononuclear cells from a healthy donor showed 18% specific lysis. These data provide additional evidence that this agent is capable of eliminating at least a subset of fresh CD7-positive peripheral blood cells. Additional studies will have to be performed to provide definitive evidence for the killing of primary leukemic blasts.

**DISCUSSION**

The central finding of this report was that the recombinant CD7-ETA scFv immunotoxin effectively killed cultured leukemic cells. Viewed from today’s state of knowledge, this may not appear unex-
pected. It was known before this study that CD7 internalizes after binding of a specific antibody and that the unmodified parental TH-69 antibody had antitumor efficacy in a xenotransplanted mouse model (26). It was further known that conventionally produced CD7-specific immunotoxins were effective against cultured leukemic cells (27–32).

Finally, recombinant scFv immunotoxins (CD22-ETA and CD25-ETA) carrying the same truncated ETA variant that was used here had been reported to be effective in patients with relapsed, chemotherapy-refractory hairy cell leukemia and CD25-positive hematological malignancies (37, 38). These latter reports established that scFv-ETA immunotoxins constructed by a similar principle as the one used here are effective against certain forms of human leukemias in vivo.

Contrasted with this background, the results of the present study offer several new elements of knowledge: (a) the construction principle of recombinant scFv-ETA molecules used here works not only for the CD22 and CD25 antigens (37, 38) but also for CD7; it may also work for a number of other cell surface antigens expressed in similar concentrations and internalized with similar kinetics after binding of an antibody; (b) several of these scFv-ETA toxins induce efficient lysis in the concentration range of ng/ml (37, 47). This is true both for the CD22-ETA scFv toxin (37) and the CD7-ETA chimeras reported here, as well as other published constructs (27, 47). Although different methods were used to quantitate cell death, and the results are not strictly comparable, it is probably still fair to state that several of these scFv-ETA toxins work with similar potency on human leukemic cells and are effective in the concentration range of <100 ng/ml. A precise comparison of the various agents can only be achieved in a side-by-side evaluation using the same assay for cellular toxicity; (c) the attachment of a truncated ETA domain did not alter the binding specificity of the scFv portion in agreement with the results reported by others (37, 38, 47); in our construct, a different linker was used than in other published constructs, and, thus, a new element of information is that the precise sequence of the linker does not appear to be critical; (d) cell death occurred by apoptosis as opposed to other mechanisms; this was shown in our study by Annexin V staining and PARP cleavage; and (e) our CD7-specific toxin worked not only for the rapidly dividing T-ALL cell lines CEM and Jurkat but also for long-term cultures of leukemia-derived HOL cells, although with lower efficiency. The reasons for the reduced efficiency are unknown, but probably the difference is attributable to the fact that stable lines differ from primary cells by the gain of additional mutations. This often leads to the selection of cells with a shorter generation time that may also be more susceptible to the agent.

The finding that ~20% of specific lysis was obtained in long-term primary cultures and cultures of fresh biopsy material as opposed to >95% in stable lines is not a disappointment. In other studies published to date, the effect of immunotoxins was evaluated primarily by using stable lines and xenograft models, in which human leukemic cells were transplanted into immunocompromised mice and were then treated with the agent (26). Studies describing the efficacy of scFv immunotoxins for primary patient cells (54) are limited. Therefore, other authors may also have experienced difficulties in evaluating such agents in primary cells. In view of these difficulties, it is encouraging that 20% of cellular lysis was reproducibly obtained in the present study in long-term primary cultures. The observation that only 20% of lysis was obtained in a 96-h period does not preclude the potential future usefulness of this agent in vivo, because in vivo it may be administered in several successive doses, and, thus, a higher percentage of killing may be achieved. The usefulness of this agent in vivo may further depend on a number of parameters that have not been evaluated in our studies with cultured cells and that can only be assessed by in vivo experiments.

In summary, in the present study, a CD7-ETA scFv immunotoxin was constructed with a design aiming at circumventing some of the problems encountered with earlier CD7-directed immunotoxins generated by chemical coupling of the toxin to the antibody. The new agent bound to CD7-positive T-lymphoid cells and killed them by the induction of apoptosis. In view of the known fact that CD7 internalizes rapidly after ligand binding and the unpublished observation that clinical application of the native unmodified TH-69 antibody leads to antigen modulation resulting in CD7-negative cells, a CD7-specific immunotoxin appears promising against T-cell malignancies. This expectation is further supported by the recent findings that other immunotoxins constructed by fusions to a truncated ETA produced encouraging clinical results. Treating patients with hairy cell leukemia with the immunotoxin BL22 (CD22-ETA), Kreitman et al. did not observe any pulmonary edema, a complication encountered after treatment with corresponding immunotoxins containing a deglycosylated ricin A chain (30, 31). The CD7-ETA scFv immunotoxin reported here may therefore circumvent some of the problems seen with older immunotoxins.

Although a CD7-positive subpopulation of CD34+CD38− cord blood cells with differentiation capabilities beyond the T-cell lineage has been described (16, 17) and the expression of CD7 on immature AML (7–10) could even be interpreted as an indication for progenitors to myeloid and T lineage, no significant CD7 expression is found on most hematopoietic progenitor cells (16). Therefore, an immunotoxin directed against CD7 has a promising range of prospective applications against T-cell neoplasms. Although a greater fraction of T-cell leukemias and some cases of AML express CD7 on their surface than CD3, CD4, CD5, or CD25, the CD7 construct still avoids eliminating too many normal T cells important for the maintenance of relevant immune functions, because it spares the CD7-negative subset of normal T-lymphoid cells (23). Elimination of a too large subset of normal T cells is a problem accompanying the clinical use of the CD52 antibody Campath-1H. Therefore, the CD7 immunotoxin reported here deserves further testing in vivo models, and such experiments are under way.

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REFERENCES


CD7-EXOTOXIN A scFv IMMUNOTOXIN

"Immunotoxins are a unique class of therapeutic agents that have become increasingly important in the treatment of cancer. This review focuses on the design, characterization, and clinical application of immunotoxins targeting the CD7 antigen, a glycoprotein involved in T-cell activation. 

The CD7 antigen, also known as the Leu-7 antigen, is a surface molecule expressed on a subset of T-cells and is highly specific for T-cell lineage differentiation. Studies have shown that CD7 plays a crucial role in the activation of the T-cell receptor complex.

Immunotoxins are created by fusing a cytotoxic protein, such as Pseudomonas exotoxin A (PE38), to a monoclonal antibody (mAb) that targets a specific antigen, in this case CD7. The antibody portion of the immunotoxin directs the cytotoxic agent to the malignant cells, while the toxin portion induces cell death.

The design of immunotoxins involves several critical steps: the selection of the appropriate antibody, the choice of the cytotoxic protein, and the optimization of their interaction. The antibody must be highly specific for the target antigen, with minimal cross-reactivity to normal tissues. The cytotoxic protein should have high specificity and potency, with minimal side effects.

Clinical trials have shown promising results with CD7-targeted immunotoxins. These trials have demonstrated safety, tolerability, and tumor response in various hematologic malignancies. However, challenges remain in improving efficacy, reducing toxicity, and optimizing delivery systems.

In conclusion, CD7-targeted immunotoxins represent a promising therapeutic strategy for the treatment of CD7-positive malignancies. Ongoing research will be crucial in refining these agents to enhance their therapeutic potential.

\[CD7-EXOTOXIN A scFv IMMUNOTOXIN\]
A Recombinant CD7-specific Single-Chain Immunotoxin Is a Potent Inducer of Apoptosis in Acute Leukemic T Cells

Matthias Peipp, Heide Küpers, Domenica Saul, et al.


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