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(54) Title: ANTI-CD7 FUSION PROTEIN

(57) Abstract: The present invention is directed to an anti-CD7 fusion protein, a nucleic acid sequence, which codes for this fusion protein and an expression vector, which comprises this nucleic acid sequence. The present invention is further directed to a method for producing of substantially pure anti-CD7 fusion protein, a therapeutic composition comprising said anti-CD7 fusion protein and its use in the treatment of disorders which involve a hyperproliferation of CD7-positive cells as well as in the prophylaxis or treatment of T-cell mediated diseases or conditions of the immune system.

ANTI-CD7 FUSION PROTEIN

DESCRIPTION

The present invention is directed to an anti-CD7 fusion protein, a nucleic acid sequence, which codes for this fusion protein and an expression vector, which comprises this nucleic acid sequence. The present invention is further directed to a method for producing of substantially pure anti-CD7 fusion protein, a therapeutic composition comprising said anti-CD7 fusion protein and its use in the treatment of disorders which involve a hyperproliferation of CD7-positive cells as well as in the prophylaxis or treatment of T-cell mediated diseases or conditions of the immune system.

In the context of this application, the abbreviations have the following meaning:

ALL: acute lymphoblastic leukemia, AML: acute myeloid leukemia, cDNA: complementary DNA, DMEM: Dulbecco's modified Eagle medium, ETA: exotoxin A, FACS: fluorescence-activated cell sorter, MCSP: melanoma-associated chondroitin sulfate proteo-glycan, PARP: poly (ADP-ribose) polymerase, PBS: phosphate buffered saline, PCR: polymerase chain reaction, PI: propidium iodide, PI3 Kinase: phosphoinositide 3-kinase, RPMI: Roswell Park Memorial Cancer Institute, RTA: ricin A toxin, scFv: single chain fragment from the variable region, T-ALL: acute T-cell leukemia.

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 immunoconjugate gemtuzumab ozogamicin (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 below 30%, and this category ranks as the worst in lymphoma prognosis (4). The CD52 antibody CAMPATH-1H (Alemtuzumab), recently approved by the FDA 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 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).

One of the prerequisites for a successful immunotherapy of T-cell neoplasias is the selection of an appropriate target antigen, which ideally should be T-cell specific and expressed on most T-cell lymphomas and leukemias, but absent on at least a portion of normal T-lymphocytes. The CD7 antigen meets these requirements. In addition, a substantial portion of AML of M1/2 type is also CD7-positive (7-10). CD7 is a cell surface glycoprotein of approximately 40 kDa and a member of the immunoglobulin superfamily (11). Galectin I is a ligand for the extracellular domain of CD7, and interaction of T-lymphoid cells with this ligand promotes apoptosis (12). Therefore, CD7 may be viewed as a novel type of "death receptor", and other specific functions of CD7 are still unknown. The protein participates in signaling processes to the inside of cells, demonstrated by its association with PI3-kinase after binding of CD7-specific antibodies (13). Upon activation of T-cells, the surface density of CD7 is increased, and CD7 has been proposed to participate in the activation and surface adhesion of mature T-cells and NK-cells (14, 15). CD7 is a marker for very early stages of T-cell maturation and is already present on hematopoietic progenitors in the fetal liver and on pluripotent progenitors of T-cells in the thymus and bone marrow. It is expressed on a majority of human thymocytes and on a large subset (approx. 85%) of peripheral blood T-cells and NK-cells (11,16-21). The remaining subset of CD7-negative peripheral T-cells maintains immune functions needed for the prevention of opportunistic infections and the engraft-

ment of hematopoietic stem cells. This subset may therefore become relevant for therapeutic purposes, because it may serve to repopulate the T-cell compartment at least in part after a CD7-directed therapy.

A key property of CD7 for therapeutic applications is its rapid internalization after binding by an antibody, even after binding by monovalent antibody fragments (22). This property makes the antigen well suited for targeting by immunotoxins which are internalized together with the antigen and subsequently poison the cell from inside.

Several investigators have targeted CD7, because the rapid internalization of the antigen after antibody binding makes this molecule attractive for the design of immunotoxins (25-28). The DA7 immunotoxin, consisting of the RTA chemically linked to a CD7-specific antibody, showed clinical efficacy in phase I trials (28,29). Chemically linked as used herein means preferably a linkage via a disulfide bond (for a general review see (51)). The use of this toxin however was limited by its instability and associated vascular toxicity.

The toxin saporin was linked via a bifunctional crosslinker and a disulfide bridge (30), and pokeweed antiviral protein was chemically linked to CD7-specific antibodies (26). In previous attempts, the inventors have 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 (31). CD7 therefore meets the requirements for a potentially useful target for immunotoxins.

However, until now known CD7-directed immunotoxins were generated by conventional chemical coupling and suffered from instability and accompanying side-effects, such as vascular toxicity.

It is therefore a problem underlying the present invention to provide an anti-CD7 protein, which is stable and does not show the above described unwanted side effects.

This problem is solved by a fusion protein, comprising the following functionally linked components: an anti-CD7 antibody or a functional part thereof and an immunotoxin, which lacks a binding specificity for receptors on mammalian cells, and homologues or fragments thereof, which retain biological activity.

Two key steps were taken in the present invention. One is the generation of a stable peptide bond between the anti CD7 antibody or the functional part thereof, for example a scFv antibody fragment and the toxin by recombinant genetics. The other is the use of a truncated toxin, which is unable to enter human cells on its own, in case it should be cleaved from the antibody moiety. This truncated toxin is devoid of its own binding domain for receptors on mammalian cells, and therefore generates significantly fewer side effects. The recombinant toxin created here can only be absorbed by CD7-positive cells by virtue of its anti CD7 portion and the CD7 internalization mechanisms of these cells.

A preferably used toxin is *Pseudomonas* ETA, specifically the truncated version lacking domain I and containing only domains II and III. 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, which causes apoptosis by inhibiting protein synthesis via a block of the translation elongation factor-2. Consequently, the truncated version of ETA lacking domain I is not toxic as long as it remains in the extracellular space. Once coupled to an antibody directed against an antigen capable of internalizing, the truncated ETA becomes a potent immunotoxin.

Generally, an anti-CD7 fusion protein of the present invention comprises the following functionally linked components: an anti-CD7 antibody or a functional part thereof and

an immunotoxin, which lacks a binding specifity for receptors on mammalian cells, and homologues or fragments thereof, which retain biological activity.

As defined herein, a „fusion protein" generally is a hybrid protein generated by fusing and expressing the protein coding regions of two or more genes.

The term "anti-CD7 antibody or a functional part thereof" refers to an amino acid sequence capable of binding to mammalian, and more preferably human, CD7 antigen on target cells.

The anti-CD7 antibody of the fusion proteins of the present invention is a polyclonal or monoclonal antibody to CD7, preferably a monoclonal anti-CD7 antibody. The term "antibody" as used in the context of the present invention comprises intact immunoglobulins as well as various forms of modified or altered antibodies, including fragments of antibodies, such as a Fv fragment, a Fab or F(ab')2 fragment, a single chain antibody, and other fragments which retain the antigen binding function and specificity of the parent antibody. The antibody can be of mammal, in particular human origin or may be chimeric or humanized. Antibodies capable of binding specifically to CD7 antigen may be produced by hybridomas prepared using well-known procedures which are based on the work of Kohler and Milstein [Nature 256: 495-97 (1975)].

The term "F(ab')2" as used herein is related to a divalent fragment of an antibody including the hinge regions and the variable and first constant regions of the heavy and light chains, which can be produced by pepsin digestion of the native antibody molecule, or by recombinant means. The F(ab')2 fragment has exactly the same antigen-binding characteristics as the original antibody.

Digestion with the protease papain cleaves antibody molecules into three fragements. Two fragments are identical, contain the antigen-binding activity and are therefore

termed "Fab" (Fragment antigen binding). The Fab fragment comprises the variable and first constant regions of the heavy and light chains.

According to one preferred embodiment, the CD7-binding moiety of the invention comprises a single chain Fv region (or CD7-binding fragment thereof) of an antibody. This is a truncated Fab comprising only the V domain of a heavy chain linked by a stretch of synthetic peptide to a V domain of a light chain.

Examples of specific anti-CD7 antibodies the whole or fragments of which are suitable to be employed as a CD7-binding domain of the invention are TH 69. The CD7 hybridoma antibody TH-69 is preferably used for the construction of a CD7-specific fusion protein, because of its high binding affinity (7.6×10^9 Mol^{-1}). Binding of this antibody to the human T-cell lines CEM, Jurkat, and MOLT-16, derived from ALL, did not affect the proliferation of these cells in culture and did not induce apoptosis to a measurable extent. However, in athymic ("nude", $nu^{-/-}$) and SCID mice xenotransplanted with CEM or MOLT-16 cells, the same antibody produced significant anti-tumor effects (24). Binding of the antibody caused a rapid downregulation ("modulation") of the antigen, and the Fc portion of the antibody was essential for the anti-tumor effect *in vivo*.

Further examples of anti CD7 antibodies, which can be used in the fusion protein of the present invention are as follows (clone names): 1-3, 3A1, 3A1e-12H7, 7G5, 8H8.1, 124-1D1 and many others. These and further examples of publicly available anti-CD7 antibodies as well as further information can be found in the HLDA Antibody Database of Martin R. Hadam, which is accessible in the internet via http:\\www.mh-hannover.de (printout enclosed).

The immunotoxin of the present invention consists preferably of domains II and III of *Pseudomonas Exotoxin* A (ETA). Further useful embodiments are domains of *diphteria*

toxin, ricin A, pokeweed antiviral protein or human pancreatic RNAse, which do not bind to receptors on mammalian cells.

The components of the present fusion protein are linked by a polypeptide linker sequence, which preferably comprises at least 20 amino acid residues. According to a preferred embodiment, the fusion protein of the present invention comprises as a linker sequence the amino acid sequence of **GGGGSGGGGSGGGGSGGGGS** (SEQ ID NO: 3) or a variant of said linker sequence, wherein said variant comprises one or more insertions, substitutions and/or deletions as compared to the sequence **GGGGSGGGGSGGGGSGGGGS,** and wherein the biological activity upon CD7 bearing cells of the said fusion protein comprising the modified linker sequence is substantially equal to the said activity of the fusion protein comprising the unmodified linker sequence SEQ ID No: 3.

According to a further embodiment, a fusion protein is provided having the amino acid sequence of SEQ ID NO: 1 or 2. The fusion proteins of SEQ ID NO: 1 and 2 only differ in the last amino acids. In SEQ ID NO: 2, the last amino acids REDLK of SEQ ID NO: 1 have been replaced by KDEL. The whole sequences of SEQ ID NO: 1 and 2 are as follows (linker sequence bold-faced):

SEQ ID NO. 1:

HHHHHHGAQPAMAAYKDIQMTQTTSSLSASLGDRVTISCSASQGISNYLNWYQ
QKPDGTVKLLIYYTSSLHSGVPSRFSGSGSGTDYSLTISNLEPEDIATYYCQQYSK
LPYTFGGGTKLEIKRGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVKPGGSL
KLSCAASGLTFSSYAMSWVRQTPEKRLEWVASISSGGFTYYPDSVKGRFTISRD
NARNILYLQMSSLRSEDTAMYYCARDEVRGYLDVWGAGTTVTVSSASGA**GGG**
GSGGGGSGGGGSGGGGSAAALEGGSLAALTAHQACHLPLETFTRHRQPRGW
EQLEQCGYPVQRLVALYLAARLSWNQVDQVIRNALASPGSGGDLGEAIREQPE
QARLALTLAAAESERFVRQGTGNDEAGAASADVVSLTCPVAAGECAGPADSGD
ALLERNYPTGAEFLGDGGDVSFSTRGTQNWTVERLLQAHRQLEERGYVFVGYH
GTFLEAAQSIVFGGVRARSQDLDAIWRGFYIAGDPALAYGYAQDQEPDARGRIR
NGALLRVYVPRSSLPGFYRTGLTLAAPEAAGEVERLIGHPLPLRLDAITGPEEEG
GRLETILGWPLAERTVVIPSAIPTDPRNVGGDLDPSSIPDKEQAISALPDYASQPG
KPPREDLK*

SEQ ID NO. 2:

HHHHHHGAQPAMAAYKDIQMTQTTSSLSASLGDRVTISCSASQGISNYLNWYQ
QKPDGTVKLLIYYTSSLHSGVPSRFSGSGSGTDYSLTISNLEPEDIATYYCQQYSK
LPYTFGGGTKLEIKRGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVKPGGSL
KLSCAASGLTFSSYAMSWVRQTPEKRLEWVASISSGGFTYYPDSVKGRFTISRD
NARNILYLQMSSLRSEDTAMYYCARDEVRGYLDVWGAGTTVTVSSASGA**GGG**
GSGGGGSGGGGSGGGGSAAALEGGSLAALTAHQACHLPLETFTRHRQPRGW
EQLEQCGYPVQRLVALYLAARLSWNQVDQVIRNALASPGSGGDLGEAIREQPE
QARLALTLAAAESERFVRQGTGNDEAGAASADVVSLTCPVAAGECAGPADSGD
ALLERNYPTGAEFLGDGGDVSFSTRGTQNWTVERLLQAHRQLEERGYVFVGYH
GTFLEAAQSIVFGGVRARSQDLDAIWRGFYIAGDPALAYGYAQDQEPDARGRIR
NGALLRVYVPRSSLPGFYRTGLTLAAPEAAGEVERLIGHPLPLRLDAITGPEEEG
GRLETILGWPLAERTVVIPSAIPTDPRNVGGDLDPSSIPDKEQAISALPDYASQPG
KPPKDEL*

The amino acid sequences of the fusion proteins of the present invention also encompass all sequence variants differing from the herein disclosed sequences by amino acid insertions, deletions, and substitutions.

Preferably, amino acid "substitutions" are the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, i.e., conservative amino acid replacements. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

"Insertions" or "deletions" are typically in the range of about 1 to 5 amino acids. The variation allowed may be experimentally determined by systematically making

insertions, deletions, or substitutions of amino acids in a polypeptide molecule using recombinant DNA techniques and assaying the resulting recombinant variants for activity. This does not require more than routine experiments for the skilled artisan.

According to a further embodiment, the present invention provides nucleic acid sequences, which code for the herein described anti-CD7 fusion proteins, including the aforementioned amino acid sequences of SEQ ID NO: 1, 2 and 3.

The term "nucleic acid sequence" refers to a heteropolymer of nucleotides or the sequence of these nucleotides. The terms "nucleic acid" and "polynucleotide" are used interchangeably herein to refer to a heteropolymer of nucleotides.

The polynucleotides of the present invention also include, but are not limited to, a polynucleotide that hybridizes to the complement of the disclosed nucleotide sequences under stringent hybridization conditions; a polynucleotide which is an allelic variant of any polynucleotide recited above; a polynucleotide which encodes a species homologue of any of the proteins recited above; or a polynucleotide that encodes a polypeptide comprising an additional specific domain or truncation of the disclosed fusion proteins.

The term "stringent" is used to refer to conditions that are commonly understood in the art as stringent. Stringent conditions can include highly stringent conditions (i. e., hybridization to filter-bound DNA under in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65 °C, and washing in 0.1xSSC/0.1% SDS at 68 °C), and moderately stringent conditions (i.e., washing in 0.2xSSC/0.1% SDS at 42 °C).

According to preferred embodiments, the present invention provides the nucleic acid sequences of SEQ ID NO: 4 and 5, which code for the fusion proteins of SEQ ID NO: 1 and 2, respectively.

SEQ ID NO: 4:

catcaccatcaccatcacggggcccagccggccatggcggcctacaaagatatccagatgacacagactacatcctccctgtc
tgcctctctgggagacagagtcaccatcagttgcagtgcaagtcagggcattagcaattatttaaactggtatcagcagaaacca
gatggaactgttaaactcctgatctattacacatcaagtttacactcaggagtcccatcaaggttcagtggcagtgggtctgggac
agattattctctcaccatcagcaacctggaacctgaagatattgccacttattattgtcagcagtatagcaagcttccgtacacgttc
ggagggggaccaagctggaaataaaacgtggtggtggtggttctggtggtggtggttctggcggcggcggctccggtggt
ggtggatccgaggtgcaactggtggagtctgggggaggcttagtgaagcctgggggtccctgaaactctcctgtgcagcct
ctggactcactttcagtagctatgccatgtcttgggttcgccagactccagagaagaggctggagtgggtcgcatccattagtag
tggtggtttcacctactatccagacagtgtgaagggccgattcaccatctccagagataatgccaggaacatcctgtatctgcaa
atgagcagtctgaggtctgaggacacggccatgtattactgtgcaagagacgaggtacgggggtacctcgatgtctggggcg
cagggaccacggtcaccgtttcctcggcctcgggggcc**GGTGGTGGCGGCAGTGGTGGTGGCG**
GCAGTGGTGGTGGCGGCAGTGGTGGTGGCGGCAGTgcggccgcgctagagggcgg
cagcctggccgcgctgaccgcgcaccaggcctgccacctgccgctggagactttcacccgtcatcgccagccgcgcggctg
ggaacaactggagcagtgcggctatccggtgcagcggctggtcgccctctacctggcggcgcgactctcatggaaccaggtc
gaccaggtgatccgcaacgccctggccagccccggcagcggcggcgacctgggcgaagcgatccgcgagcagccggag
caggcccgtctggccctgaccctggccgccgccgagagcgagcgcttcgtccggcagggcaccggcaacgacgaggccg
gcgcggccagcgccgacgtggtgagcctgacctgcccggtcgccgccggtgaatgcgcgggcccggcggacagcggcg
acgccctgctggagcgcaactatcccactggcgcggagttcctcggcgacggtggcgacgtcagcttcagcacccgcggca
cgcagaactggacggtggagcggctgctccaggcgcaccgccaactggaggagcgcggctatgtgttcgtcggctaccacg
gcaccttcctcgaagcggcgcaaagcatcgtcttcggcggggtgcgcgcgcgcagccaggatctcgacgcgatctggcgcg
gtttctatatcgccggcgatccggcgctggcctacggctacgcccaggaccaggaacccgacgcgcgcggccggatccgca
acggtgccctgctgcgggtctatgtgccgcgctcgagcctgccgggcttctaccgcaccggcctgaccctggccgcgccgga
ggcggcgggcgaggtcgaacggctgatcggccatccgctgccgctgcgcctggacgccatcaccggccccgaggaggaa
ggcgggcgcctggagaccattctcggctggccgctggccgagcgcaccgtggtgattccctcggcgatccccaccgacccg
cgcaacgtcggcggcgacctcgacccgtccagcatccccgacaaggaacaggcgatcagcgccctgccggactacgccag
ccagcccggcaaaccgccgcgcgaggacctgaagtaa

SEQ ID NO: 5:

catcaccatcaccatcacggggcccagccggccatggcggcctacaaagatatccagatgacacagactacatcctccctgtc
tgcctctctgggagacagagtcaccatcagttgcagtgcaagtcagggcattagcaattatttaaactggtatcagcagaaacca
gatggaactgttaaactcctgatctattacacatcaagtttacactcaggagtcccatcaaggttcagtggcagtgggtctgggac
agattattctctcaccatcagcaacctggaacctgaagatattgccacttattattgtcagcagtatagcaagcttccgtacacgttc
ggagggggaccaagctggaaataaaacgtggtggtggtggttctggtggtggtggttctggcggcggcggctccggtggt
ggtggatccgaggtgcaactggtggagtctgggggaggcttagtgaagcctgggggtccctgaaactctcctgtgcagcct
ctggactcactttcagtagctatgccatgtcttgggttcgccagactccagagaagaggctggagtgggtcgcatccattagtag
tggtggtttcacctactatccagacagtgtgaagggccgattcaccatctccagagataatgccaggaacatcctgtatctgcaa
atgagcagtctgaggtctgaggacacggccatgtattactgtgcaagagacgaggtacgggggtacctcgatgtctggggcg
cagggaccacggtcaccgtttcctcggcctcgggggcc**GGTGGTGGCGGCAGTGGTGGTGGCG**
GCAGTGGTGGTGGCGGCAGTGGTGGTGGCGGCAGTgcggccgcgctagagggcgg
cagcctggccgcgctgaccgcgcaccaggcctgccacctgccgctggagactttcacccgtcatcgccagccgcgcggctg
ggaacaactggagcagtgcggctatccggtgcagcggctggtcgccctctacctggcggcgcgactctcatggaaccaggtc
gaccaggtgatccgcaacgccctggccagccccggcagcggcggcgacctgggcgaagcgatccgcgagcagccggag
caggcccgtctggccctgaccctggccgccgccgagagcgagcgcttcgtccggcagggcaccggcaacgacgaggccg

gcgcggccagcgccgacgtggtgagcctgacctgcccggtcgccgccggtgaatgcgcgggcccggcggacagcggcg
acgccctgctggagcgcaactatcccactggcgcggagttcctcggcgacggtggcgacgtcagcttcagcacccgcggca
cgcagaactggacggtggagcggctgctccaggcgcaccgccaactggaggagcgcggctatgtgttcgtcggctaccacg
gcaccttcctcgaagcggcgcaaagcatcgtcttcggcggggtgcgcgcgcgcagccaggatctcgacgcgatctggcgcg
gtttctatatcgccggcgatccggcgctggcctacggctacgcccaggaccaggaacccgacgcgcgcggccggatccgca
acggtgccctgctgcgggtctatgtgccgcgctcgagcctgccgggcttctaccgcaccggcctgaccctggccgcgccgga
ggcggcgggcgaggtcgaacggctgatcggccatccgctgccgctgcgcctggacgccatcaccggccccgaggaggaa
ggcgggcgcctggagaccattctcggctggccgctggccgagcgcaccgtggtgattccctcggcgatccccaccgacccg
cgcaacgtcggcggcgacctcgacccgtccagcatccccgacaaggaacaggcgatcagcgccctgccggactacgccag
ccagcccggcaaaccgccgaaggacgagctgtaa

or a variant thereof, wherein the variant is defined as having one or more substitutions, insertions and/or deletions as compared to the sequences of SEQ ID NO: 4 or 5, provided that the said variant hybridizes under moderately stringent conditions, as defined hereinabove, to the nucleic acid sequence of SEQ ID NO: 4 or 5, and further provided that said variant codes for a protein with cytotoxic activity on CD7-bearing cells.

The present invention further comprises the nucleic acid sequence of SEQ ID NO: 6, which codes for the linker sequence of SEQ ID NO: 3:

GGTGGTGGCGGCAGTGGTGGTGGCGGCAGTGGTGGTGGCGGCAGTGG TGGTGGCGGCAGT (SEQ ID NO: 6)

or a variant thereof, wherein the variant is defined as having one or more substitutions, insertions and/or deletions as compared to the sequence of SEQ ID No. 6, provided that the said variant hybridizes under moderately stringent conditions, as defined hereinabove, to the nucleic acid sequence of SEQ ID No. 6, and further provided that the biological activity upon CD7 bearing cells of the said fusion protein comprising the modified linker sequence is substantially equal to the said activity of the fusion protein comprising the unmodified linker sequence SEQ ID No. 6.

The present invention further provides recombinant constructs comprising one of the

above described nucleic acids or a fragment thereof. The recombinant constructs of the present invention comprise a vector, such as a plasmid or viral vector, into which a nucleic acid having one of the above mentioned sequences or a fragment thereof is inserted, in a forward or reverse orientation.

According to a further aspect of the present invention, an expression vector is provided, which comprises a nucleic acid sequence coding for an anti-CD7 fusion protein. This expression vector preferably comprises one or more regulatory sequences. The term "expression vector" generally refers to a plasmid or phage or virus or vector, for expressing a polypeptide from a DNA (RNA) sequence. An expression vector can comprise a transcriptional unit comprising an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, promoters or enhancers, (2) a structural or coding sequence which is transcribed into mRNA and translated into protein, and (3) appropriate transcription initiation and termination sequences. Structural units intended for use in yeast or eukaryotic expression systems preferably include a leader sequence enabling extracellular secretion of translated protein by a host cell. Alternatively, where recombinant protein is expressed without a leader or transport sequence, it may include an N-terminal methionine residue. This residue may or may not be subsequently cleaved from the expressed recombinant protein to provide a final product.

The present invention further provides host cells which has been transformed to contain the polynucleotides of the invention. The term "transformation" means introducing DNA into a suitable host cell so that the DNA is replicable, either as an extrachromosomal element, or by chromosomal integration.

For example, such host cells may contain nucleic acids of the invention introduced into the host cell using known transformation methods. The present invention still further provides host cells genetically engineered to express the polynucleotides of the invention, wherein such polynucleotides are in operative association with a regulatory

sequence heterologous to the host cell which drives expression of the polynucleotides in the cell.

The host cell can be a higher eukaryotic host cell, such as a mammalian cell, a lower eukaryotic host cell, such as a yeast cell, or can be an insect cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the recombinant construct into the host cell can be effected by calcium phosphate transfection, DEAE, dextran mediated transfection, or electroporation (Davis, L. et al., Basic Methods in Molecular Biology (1986)).

The most preferred cells are those which do not normally express the particular polypeptide or protein or which expresses the polypeptide or protein at low natural level. Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., in Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, New York (1989).

The mammalian cell is pereferably a CHO, COS, HeLa, 293T, HEH or BHK cell. As procaryotic cells, *E.coli*, *Bacillus subtilis*, or bacterial cells of the genus of *Streptomyces* or *Proteus mirabilis L* –forms are preferred.

The invention also relates to methods for producing a substantially pure fusion protein comprising growing a culture of the cells of the invention in a suitable culture medium, and purifying the protein from the culture. For example, the methods of the invention include a process for producing a polypeptide in which a host cell containing a suitable expression vector that includes a polynucleotide of the invention is cultured under conditions that allow expression of the encoded polypeptide. The polypeptide can be recovered from the culture, conveniently from the culture medium, and can be further purified. The resulting expressed protein may for example be purified from such culture

(i.e., from culture medium or cell extracts) using known purification processes, such as gel filtration and ion exchange chromatography.

According to a further aspect, the present invention provides a therapeutic composition, comprising a therapeutically effective amount of one or more of the fusion proteins or nucleic acids as disclosed herein and a pharmaceutically acceptable carrier.

Such a composition may also contain (in addition to the ingredient and the carrier) diluents, fillers, salts, buffers, stabilizers, solubilizers and other materials well known in the art. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredient(s). The characteristics of the carrier will depend on the route of administration. The therapeutic composition may further contain other agents which either enhance the activity or use in treatment. Such additional factors and/or agents may be included in the therapeutic composition to produce a synergistic effect or to minimize side-effects.

Techniques for formulation and administration of the compounds of the present application may be found in "Remington's Pharmaceutical Sciences", Mack Publishing Co., Easton, PA, latest edition.

The compositions contain a therapeutically effective amaount or dose of the respective ingredient. A therapeutically effective dose refers to that amount of the compound sufficient to result in amelioration of symptoms, e.g., treatment, healing, prevention or amelioration of such conditions. Suitable routes of administration may, for example, include parenteral delivery, including intramuscular and subcutaneous injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal injections. Intravenous administration to the patient is preferred.

A typical composition for intravenous infusion can be made up to contain 250 ml of sterile Ringer's solution, and 10 mg of the ingredient. See Remington's Pharmaceutical

Science (15th Ed., Mack Publishing Company, Easton, Ps., 1980). Preferably, the therapeutic composition of the present invention is a vaccine.

The compositions of the present invention may be used for the treatment of disorders, involving a hyperproliferation of CD7-positive cells, e.g. in the treatment of CD7 positive acute T-cell and/or myeloid leukemias.

Furthermore, they may be of use for the prophylaxis or treatment of Graft-versus-host disease (GvHD).

The present invention further provides a method for the prophylaxis or treatment of T-cell mediated diseases or conditions by administering fusion protein to a patient in vivo for the purpose of systemically killing T cells in the patient. The method comprises administering to an animal in need thereof a T-cell depleting effective amount of a fusion protein of the present invention in a dosage effective to substantially deplete the T-cells in said animal. Depletion of the levels of T cells in the bone marrow, the peripheral blood and/or lymphoid tissues of the animal can ameliorate the T-cell mediated response to an antigen, and assist in tolerance induction. For example, the immunotoxins may be administered to prevent a graft rejection in an animal that is a recipient of an allotransplant (or xenotransplant).

The physician in any event will determine the actual dosage which will be most suitable for an individual patient and will vary with the age, weight and response of the particular patient. There can, of course, be individual instances where higher or lower dosage ranges are merited, and such are within the scope of this invention.

The immunotoxins can be administered in vivo either alone or in combination with other pharmaceutical agents effective in treating acute or chronic transplant rejection.

A method of conditioning an animal to be transplanted with cells, or a tissue or organ of a donor, comprises the steps of (a) depleting the CD7-bearing cell population in the animal; (b) providing an transplant comprising isolated bone marrow and/or stem-cell enriched peripheral blood cells of the donor, wherein the CD7-bearing cell population in a) and/or the transplant in b) are treated with a T-cell depleting effective amount of a fusion protein of the present invention; and (c) introducing the transplant into the animal.

According to a further aspect, the present invention comprises a method for treating disorders, which involve a hyperproliferation of CD7-positive cells, in an animal, comprising: administering to an animal in need of such treatment, a composition of the present invention in a dosage effective to substantially eliminate the hyperproliferating CD7-positive cells in said animal.

The present invention is now further illustrated and described in more detail by the following drawings and the Examples. In both, the aspect of a scFv-ETA fusion protein is presented, however, it is noted that the scope of the present invention is not limited to this specific embodiment.

Figure legends

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 non-related 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.

Fig. 2

Construction scheme for the recombinant immunotoxin. 6xHis: N-terminal hexa-histidine tag; V_L, V_H: variable regions of the L- and H-chains of the TH-69 monoclonal antibody. Linkers 1, 2: flexible linkers of glycine and valine residues (Examples); Exotoxin A': truncated exotoxin A fragment, consisting of domains II and III, (amino acids 253-613) but lacking the receptor-binding domain I of the intact Pseudomonas toxin. Molecular masses of the various fragments in Da were calculated from their amino acid sequence.

Fig.3

ScFv fusion proteins CD7-ETA and MCSP-ETA are capable of binding to antigen-positive cells. Cells were stained with purified scFv-ETA fusion proteins (black) or with a nonrelated scFv-ETA fusion protein (white) at the same concentration and analyzed by FACS. (A): CD7-positive CEM cells stained with CD7-ETA. (B): CD7-negative SEM cells stained with CD7-ETA. (C) MCSP-positive LLM cells stained with MCSP-ETA, (D) MCSP-negative MV4-11 cells stained with MCSP-ETA.

Fig.4

Killing by CD7-ETA is blocked specifically by the parental TH-69 antibody. CEM cells were left untreated (○), or were treated with 100 ng/ml CD7-ETA (◇), 100 ng/ml CD7-ETA + TH-69 (ρ), 100 ng/ml CD7-ETA + isotype control antibody (π), TH-69 (□), isotype antibody (◆). At given timepoints viable cells were counted by trypan-blue staining. Each timepoint was measured in triplicates and standard deviations are indicated by error bars. The data are representative of four separate experiments.

Fig.5

CD7-ETA induces cell death of CEM leukemic blasts at low concentrations. Antigen-positive CEM cells (A) and antigen-negative SEM cells (B) were left untreated (◆), or

were treated with various concentrations of CD7-ETA: 1 ng/ml (■), 5 ng/ml (π), 10 ng/ml (□), 50 ng/ml (●), 100 ng/ml (○), 500 ng/ml (+), 1000 ng/ml (—). Aliquots of cells were evaluated for percentage of cell death by hypotonic PI staining and flow cytometry (FACS). Data points are mean values from three independent experiments. Standard deviations are indicated.

Fig.6

CD7-ETA induces cell death of CD7-positive Jurkat T-cells but not of CD7-negative REH and NALM-6 cells. Jurkat (○), REH (□), and Nalm-6 (●) cells were treated with 100 ng/ml CD7-ETA. Aliquots of cells were analyzed for percentage of cell death by hypotonic PI staining and FACS. Data points are the arithmetic means from three independent experiments. Standard deviations are given.

Fig.7

CD7-ETA induces apoptosis in CEM and Jurkat cells. (A) Jurkat and CEM cells were treated with 100 ng/ml CD7-ETA or MCSP-ETA as a negative control. Cells were stained with annexin V and PI (in PBS). Numbers in the bottom right quadrant of each plot represent the percentage of cells in early apoptosis (annexin V-positive and PI-negative). The data are representative of three different experiments. (B) Aliquots of CD7-ETA and MCSP-ETA treated cells were analyzed for cleavage of PARP (substrate of caspase-3) by western blot. The specific cleavage product of 85 kDa was only detectable in CD7-ETA treated samples.

Fig. 8

Comparison of two different methods for the measurement of cell-death. A: Flow cytometric analysis of DNA content by PI staining of nuclei. Using this method, 81% cell death was obtained for the same sample that gave rise to 97.8% with the second method (B). B: measurement by annexin V and PI staining and FACS analysis. Aliquots from the same batch of treated cells were analyzed with both methods. Using method 2

(annexin and PI staining) the combined number of events in the bottom and top right panels was 97.8%.

Fig. 9

Killing by the CD7-ETA scFv toxin is antigen specific as determined by competition with the parental TH-69 hybridoma antibody and appropriate controls. Top two panels: CEM cells treated with the CD7-ETA toxin in a single dose of 100 ng/ml analyzed after 40 and 64 h of exposure to the agent; middle two panels: cells treated with the same amount of the CD7-ETA toxin plus a 100-fold molar excess of the parental TH-69 antibody, for 40 and 64 h, respectively. Bottom two panels: cells treated with the same amount of the agent plus a 100-fold molar excess of a non-relevant isotype matched control antibody for 40 and 64 h, respectively.

Fig. 10

Long-term primary cultures of leukemia-derived cells can be killed specifically by the CD7-ETA immunotoxin. HOL cells at generation 30 were treated with a first dose (100 ng/ml) of the CD7-ETA immunotoxin or the anti MCSP-ETA control toxin at time 0 and a second dose at 48 h. The percentage of dead cells was determined by annexin V and PI-staining and FACS analysis. (◆): treated with the CD7-ETA toxin alone; (X): treated with the CD7-ETA toxin plus a 100-fold molar excess of an isotype-matched control antibody; (π): treated with CD7-ETA toxin plus a 100-fold molar excess of the parental TH-69 antibody; (■): treated with the anti MCSP-ETA toxin alone. Numerical values plotted are the arithmetic means from two independent experiments. Error bars represent the standard deviations from the mean as calculated by the Microsoft Excel ™ computer program.

Examples

Bacterial strains and plasmids. *E. 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, UK) for the screening of antibody libraries. Libraries were generated in the phagemid vector pAK100 and pAK400 was employed for expression of soluble scFvs (36). *E.coli* BL21 (DE3; Novagen Inc., Madison, USA) served for the expression of scFv-ETA fusion proteins.

Culture of eukaryotic cells. Leukemia-derived SEM cells (37), CEM cells (38), Jurkat cells (39), REH cells (DSMZ, German Collection of Microorganisms and Cell Lines; Braunschweig; Germany), Nalm-6 cells (DSMZ), MV4;11 cells (American Type Culture Collection; ATCC, Rockville, Maryland) and the two hybridomas TH-69 (23) and 9.2.27 (anti-MCSP; ref. 40) were cultured in RPMI 1640-Glutamax-I medium (Invitrogen, Karlsruhe, Germany) containing 10% fetal calf serum and penicillin and streptomycin at 100 U/ml and 100 µg/ml, respectively. COS monkey cells (DMSZ, Braunschweig) were maintained in DMEM-Glutamax-I medium containing 10% fetal calf serum, penicillin and streptomycin.

Patient derived leukemic cells. HOL cells were derived from a 10 year old patient, diagnosed with precursor T-ALL (CD1a+, CD2+, CD4+, CD7+, CD8+, CD3-) at the Childrens Hospital of the University of Erlangen. He was treated according ALL-BFM-86 protocol and reached remission at day 33 of protocol I. He has been in continuous complete remission for more than 10 years after therapy. The culture was established from peripheral blood, and the cells proliferated with a doubling time of 3 days. They were cultured in Iscove's Modified Dulbecco Medium (Gibco BRL), supplemented with human transferrin and linoleic acid at 3 µg/ml, bovine serum albumin at 10 mg/ml, α-thioglycerol at 50 mg/ml, bathocuproin-disulfonic acid at 20 nM, insulin at 1 µg/ml, and

recombinant human IL-7 (Strathmann, Inc., Hamburg, Germany) at 10 ng/ml. 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.

Preparation of scFv phage display libraries. Total RNA was prepared with Trizol (Invitrogen, Karlsruhe, Germany) 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 (36). PCR amplification of immunoglobulin variable region cDNAs and cloning into the phagemid vector pAK100 was performed as described (36,41). Propagation of combinatorial scFv libraries and preparation of filamentous phages was performed following published procedures (41).

Panning of phage display libraries with intact cells. Panning of phage display libraries with intact cells was carried out using CD7-positive CEM cells and COS cells positive for the MCSP antigen. For this purpose, $5x10^6$ cells were incubated in phosphate buffered saline (PBS; Invitrogen, Grohningen, Netherlands) containing 2% nonfat dry milk (NM-PBS) to block nonspecific binding sites and then incubated with 500μl 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 50μg 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 cells. Twenty ml of 2xYT medium (16 g/liter tryptone/peptone (Roth, Karlsruhe, Germany); 10 g/liter yeast extract (Roth) and 5 g/liter NaCl) containing 1% glucose and chloramphenicol at 30 μg/ml were 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. Seventy ml of 2xYT medium were then added and isopropylthiogalactoside (IPTG) was added at a final concentration of 0.5 mM. Two hours

after infection, kanamycin was added at a final concentration of 25 μg/ml and the culture was grown overnight at 30 °C. On the following day, phages were prepared as described (41). After two rounds of panning, individual phages were purified and the inserts were sequenced (42) 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 Cambridge, UK). Expression and purification of CD7- and MCSP-specific scFv fragments was performed as described (41).

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-promotor, the plasmid pet27b(+) (Novagen) was modified as follows. To introduce a 6xHis-tag at the N-terminus and a SfiI-site, two oligonucleotides, oligo 1: 5′-cc cat cac cat cac cat cac ggg gcc cag ccg gcc g-3′; and oligo 2: 5′-ga tcc ggc cgg ctg ggc ccc gtg atg gtg atg gtg atg gg-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 HindIII/NotI digested modified pet27b(+) using oligonucleotides 3 and 4 (oligo 3: 5′ - agc ttg gcc tcg ggg gcc ggt ggt ggc ggc agt ggt ggt ggc ggc agt ggt ggt ggc ggc agt ggt ggt ggc ggc agt gc - 3′; oligo 4: 5′ - ggc cgc act gcc gcc acc acc act gcc gcc acc acc act gcc gcc acc acc act gcc gcc acc acc ggc ccc cga ggc ca - 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/Cel II and a DNA fragment encoding the truncated ETA was amplified by polymerase chain reaction (PCR) from

plasmid pSW202 (43) using the primers oligo 5: 5′ - gat cgc ggc cgc gct aga ggg cgg cag cct gg – 3′; and oligo 6: 5′- cac tag gct cag cgc ggc agt tac ttc agg tcc tcg – 3′). The amplified DNA was then ligated into the vector and sequenced.

The scFv-ETA fusion proteins were expressed under osmotic stress as described (44). Induced cultures were harvested 16-20 h after induction. The bacterial pellet from a 1 liter culture was resuspended in 10 ml of lysis-buffer (50 mM NaH_2PO_4; 300 mM NaCl; 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 3x for 1 min at 120 W in a sonicator/cell disruptor (B.Braun Biotech, Melsungen, Germany). The scFv-ETA fusion proteins were enriched by affinity chromatography using nickel-nitrilotriacetic (Ni-NTA) agarose Beads (Quiagen, Hilden, Germany) according to manufacturer's instructions.

Flow Cytometric Analysis. The binding of scFvs to cells was analyzed using a FACSCalibur FACS and the CellQuest software (Becton Dickinson, Mountain View, CA, USA). Cells were stained with scFv fragments as described (41). 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, $5x10^5$ cells were incubated for 30 min on ice with 20 µl of the immunotoxin at a concentration of 1 µg/ml. A non-related immunotoxin served as a control for background staining. The cells were washed with PBA buffer containing PBS, 0.1% bovine serum albumin, 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 pig anti-rabbit-FITC conjugate (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 $2.5x10^5$/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[3] as described (45, 46). The extent of cell death was determined by measuring the fraction of nuclei with sub-diploid DNA content. Fifteen thousand events were collected for each sample analyzed for sub-diploid nuclear DNA content. To determine whether cell death was attributable to apoptosis, whole cells were stained with fluorescein-isothiocyanate (FITC)-conjugated annexin V (Pharmingen, Heidelberg; Germany; ref. 47) and PI in PBS according to the manufacturer's protocol. For blocking experiments, cells were seeded at $2.5x10^5$/ml in 24 well plates and a 100-fold molar excess of the parental antibody or a non-related 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 (48). Western blots were performed with secondary antibodies coupled to horseradish peroxidase (Dianova, Hamburg, Germany; 49). ECL reagents (Amersham Pharmacia, 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 exotoxin A polyclonal antibody (Sigma). Full-length PARP and its specific cleavage product were detected using a mouse anti-human PARP-antibody (Pharmingen).

The recombinant CD7-specific scFv fragment and the derived immunotoxin retain specific binding. Two scFv fragments, one directed against CD7, the other specific for the MCSP antigen, were subcloned from the corresponding hybridoma cell line. For this purpose, the variable regions from the light and the heavy chain were PCR amplified and connected via overlap-extension PCR. The scFv fragments were then ligated into the phagemid vector pAK100. The obtained phage display libraries were used to identify specific binders by screening on antigen-positive cells over two rounds. After the second round of panning, individual clones were analyzed by cellular ELISA. Clones that showed the strongest binding to antigen-positve cells were subcloned into the expression vector pAK400. This vector is designed for the expression of soluble scFv fragments. Purified scFv fragments from periplasmic extracts were functionally analyzed for binding by FACS. The recombinant CD7-specific scFv fragment subcloned 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-positve 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 an scFv fragment did not detectably alter the binding specificity of the antigen-combining site.

A scheme for the construction of the recombinant immunotoxin is given in Fig. 2. The protein was purified by affinity chromatography to greater than 90% purity and approximately 100 µg of purified protein were routinely 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 due to their common design rather than their particular scFv-moiety. To this effect, the hybridoma 9.2.27-derived antibody specific for the MCSP antigen (40), 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 bound to LLM cells, a leukemia-derived pro-B cell line with chromosomal translocation t(4;11) to the MLL gene (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 sub-diploid 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 5 ng/ml and maximum death was reached at approximately 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 administration 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 non-specifically, 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 prove, that the observed cell death was due to apoptosis, CEM and Jurkat cells, were treated with CD7-ETA or with the control toxin, and then stained with annexin V and PI. Forty eight h after exposure, 30% of CEM cells and 24% Jurkat cells stained positive for annexin V and negative for PI (lower right quadrant), a characteristic sign of death by apoptosis (Fig. 7A). As a control, the recombinant anti-MCSP-ETA scFv immunotoxin of similar design failed to induce annexin staining and cell death. As a further 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 induced cell death via apoptosis for both the CEM and Jurkat lines (Fig. 7A, B).

A direct comparison of the two methods used to quantitate cell death was made. Ninety-six hours after addition of 100 ng/ml toxin, cell death was measured either by staining with propidium iodide in hypotonic solution (method 1; Fig. 8A) or by staining with propidium iodide (PI uptake) and annexin V (method 2; Fig. 8B). Method 1 showed 81.2 % of dead cells (sub-diploid DNA content), whereas measurement by staining with propidium iodide (PI uptake) plus annexin V (method 2) showed $\geq$ 97% of dead cells. Thus, measurement by method 1 under-estimated the number of dead cells relative to determination by microscopic counting and method 2 (Fig. 8A,B). Apoptotic death induced by

the toxin and evidenced by method 2 was specifically inhibited 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; upper two panels). These results confirm the conclusion that killing was antigen-specific, and that induction of apoptosis was due to receptor-mediated uptake of the immunotoxin.

The recombinant CD7-ETA immunotoxin promotes specific lysis of primary leukemic cells. To be of therapeutic interest, the toxin needs to lyse not only stable leukemia-derived cell lines but also primary leukemic cells. For this purpose, a low-passage culture of cells derived from a pediatric patient (HOL) with an acute T-cell leukemia was used. HOL cells had been kept in culture for less than 30 generations. Their generation time was 3 days as opposed to 22-24 hrs 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). Approximately 20% specific death (annexin V positive) 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. The anti-MCSP-ETA scFv immunotoxin had no effect on HOL cells, which are MCSP-negative. The agent was therefore effective not only on rapidly dividing leukemia-derived cell lines, but to a lesser degree also on long term primary cultures. It therefore merits further evaluation of its potential as a therapeutic agent.

The central finding of this invention is that the recombinant CD7-ETA scFv immunotoxin effectively killed cultured leukemic cells. Viewed from the state of the art, this appears unexpected.

Contrasted against this prior art, the results of the present study offer several new elements of knowledge. The attachment of a truncated ETA domain did not alter the binding specificity of the scFv portion. Further, the cell death occured by apoptosis as opposed to other mechanisms. This was shown in this application by annexin staining and PARP-cleavage. Finally, the CD7-specific toxin of the present invention 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 due 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.

Similar results were also obtained with freshly drawn peripheral blood cells from an adult patient treated for CD7-positive T-ALL at our University Hospital. The cells were placed in culture within 3 h after collection and an aliquot was used for FACS analysis. The patients peripheral blood contained 23% blasts before exposure to the agent. The agent was added in a single dose of 100 ng/ml and cell death was measured by FACS using annexin staining and propidium iodide uptake. After 24 h, 12% specific cell death was observed. After 48 h a total of 20% specific lysis was reached. After exposure for 48 h, the population was re-analyzed by FACS and still contained 23% blasts. Therefore, the relative proportion of blasts in the population was unaltered by the treatment. Identically treated fresh peripheral blood mononuclear cells from a healthy donor showed 18% specific lysis. These data provide a first indication that this agent is capable of affecting normal peripheral blood T cells and possibly some of the leukemic blasts.

In summary, a anti-CD7 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 lead to antigen modulation resulting in CD7-negative cells, a CD7-specific immunotoxin is a promising therapeutic agent. The anti-CD7 fusion protein reported here may therefore circumvent some of the problems seen with older immunotoxins and has a promising range of prospective applications against T-cell neoplasms. While more T-cell leukemias and lymphomas (and even some AML) express CD7 than CD25, our construct avoids targeting too many normal T-cells important for the maintenance of certain immune functions, a problem seen with the CD52 antibody Campath-1H.

Antibodies assigned to CD7

This listing contains all monoclonal antibodies assigned during HLDA1 to HLDA6

WS	Sect	WS Code	*Clone Name*	Originator	Isotype	Spec
3	T	548	1-3	Dupont	IgG2a	M
2	T	44	1-3	Dupont	IgG2a	M
6	T	6T-CD7.4	3A1	Coulter	IgG2b	M
1	T	32	3A1	Haynes	IgG1	M
5	T	T-027	3A1a	Haynes	IgG1	M
5	T	T-CD07.08	3A1a	Haynes	IgG1	M
5	BP	BP426	3A1a	Haynes	IgG1	M
3	T	549	3A1a	Haynes	IgG1	M
2	T	39	3A1a	Haynes	IgG1	M
2	T	40	3A1a	Haynes	IgG1	M
3	T	550	3A1b-4G6	Haynes	IgG2	M
2	T	41	3A1b-4G6	Haynes	IgG2	M
3	T	101	3A1d-14C2	Haynes	IgG1	M
3	T	551	3A1d-14C2	Haynes	IgG1	M
5	T	T-CD07.09	3A1e-12H7	Haynes	IgG2b	M
3	T	552	3A1e-12H7	Haynes	IgG2b	M
3	T	553	3A1f-13D1	Haynes	unknown	M
3	T	554	3A1g-14F12	Haynes	unknown	M
2	T	43	4A	Dupont	IgG2a	M

1	T	33	4A	Morishima Y	IgG2a	M
4	T	152	7G5	Lazarovits	IgG2a	
3	T	557	8H8.1	Mawas	IgG2a	M
2	T	38	8H8.1	Mawas	IgG2a	M
3	T	545	121	Bernard	unknown	
4	T	155	124-1D1	Vilella	IgG1	
4	T	154	142-9	Vilella	IgG1	
5	T	T-CD07.07	142-24	Vilella	IgG1	
4	T	153	142-24	Vilella	IgG1	
4	T	157	B-F12	Wijdenes	IgG2a	
6	T	6T-024	BE57	Boumsell	unknown	M
4	T	164	CD7-6B7	Knapp	IgG2a	M
3	T	558	CL1.3	Morishima	IgG2a	M
1	T	60	CL1.3	Morishima	IgG2a	M
3	T	561	CLB-3A1/1	Tetteroo	unknown	M
3	T	559	F93-8A6 (S-T2)	Poncelet	unknown	
4	T	161	F101-229	Carriere	IgG1	
3	T	102	G3-7	Ledbetter	IgG1	M
3	T	555	G3-7	Ledbetter	IgG1	M
2	T	30	G3-7	Ledbetter	IgG1	M
5	T	T-CD07.03	HIT7	Chen	IgG1	
5	T	T-CD07.06	HN16.42	Sun	IgG1	M
4	T	165	HuLym2	McKenzie	IgG2a	

4	T	159	I21	Boumsell	IgG2b	M
2	T	45	I21	Bernard	IgG2b	M
3	T	546	I263	Bernard	unknown	
3	T	547	I458	Bernard	unknown	
5	T	T-CD07.10	ICO-87	Baryshnikov	IgG1	
4	T	201	JOR-T7	Amador	IgM	
6	T	6T-R7	Leu-9	Becton Dickinson	IgG2ak	M
6	T	6T-CD7.2	Leu-9	Becton Dickinson	IgG2ak	M
6	BP	543	Leu-9	Becton Dickinson	IgG2ak	M
6	BP	494	Leu-9	Becton Dickinson	IgG2ak	M
5	NK	NK12	Leu-9	Becton Dickinson	IgG2ak	M
3	T	563	Leu-9	Warner	IgG2ak	M
2	T	31	Leu-9	Warner	IgG2ak	M
5	T	T-CD07.04	LN67	Sun	IgG2b	M
5	T	T-CD07.02	LS93	Sun	IgG2b	M
6	T	6T-011	LT7	Filatov	IgG2a	M
6	T	6T-CD7.1	M-T701	PharMingen	IgG1k	M
4	T	163	M-T701	Rieber	IgG1k	M
6	T	6T-015	MEM-186	Horejsi	IgG1	M
3	T	560	OKT16	Talle	unknown	
4	A	77	PANL7D	Martone	IgG2a	
4	T	160	PANL7D	Mortone	IgG2a	

5	T	T-CD07.05	SN210	Sun	IgG1	M
6	T	6T-CD7.3	T55	Nichirei	IgG2a	M
3	T	556	T55	Maeda	IgG2a	M
2	T	54	T55	Maeda	IgG2a	M
4	T	156	TH-69	Gramatzki	IgG1	M
3	T	562	Tp40	Ueda	unknown	
3	T	564	Tü14	Wernet	IgG3	M
1	T	56	Tü14	Ziegler A	IgG3	M
4	T	158	Tü93	Ziegler	IgG	M
5	T	T-163	WM31	Favaloro	IgG3	M
5	T	T-CD07.01	WM31	Favaloro	IgG3	M
4	T	35	YTH3.2	Waldmann H	IgG2b	R
3	T	142	YTH3.2	Waldmann H	IgG2b	R
4	T	162	YTH30.15	Waldmann H	IgG2b	R

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P15147

PATENT CLAIMS

1. A fusion protein, comprising the following functionally linked components:
   a) an anti-CD7 antibody or a functional part thereof; and
   b) an immunotoxin, which lacks a binding specifity for receptors on mammalian cells,

   and homologues or fragments thereof, which retain biological activity.

2. The fusion protein of claim 1, wherein the functional part of the anti-CD7 antibody comprises the scFv fragment.

3. The fusion protein of claim 1 or 2, wherein the anti-CD7 antibody is TH 69.

4. The fusion protein of one or more of the preceding claims, wherein the immunotoxin consists of domains II and III of Pseudomonas Exotoxin A (ETA).

5. The fusion protein of one or more of claims 1-3, wherein the immunotoxin consists of domains of diphteria toxin, ricin A, pokeweed antiviral protein or human pancreatic RNAse, which do not bind to receptors on mammalian cell.

6. The fusion protein of one or more of the preceding claims, wherein the components
   a) and b) are linked by a polypeptide linker sequence.

7. The fusion protein of claim 6, wherein the linker sequence comprises at least 20 amino acid residues.

8. The fusion protein of claim 6 wherein the linker comprises the sequence SEQ ID No. 3 or a variant of said linker sequence, wherein said variant comprises one or more insertions, substitutions and/or deletions as compared to the sequence of SEQ ID No. 3, and wherein the biological activity upon CD7 bearing cells of the said fusion protein comprising the modified linker sequence is substantially equal to the said activity of the fusion protein comprising the unmodified linker sequence SEQ ID No. 3.

9. A fusion protein having the amino acid sequence of SEQ ID No. 1 or 2 or a variant thereof.

10. A nucleic acid sequence, which codes for a fusion protein of one or more of the preceding claims.

11. A nucleic acid sequence which is SEQ ID No. 4 or 5 or variants thereof, wherein the variants are each defined as having one or more substitutions, insertions and/or deletions as compared to the sequence of SEQ ID No. 4 or 5, provided that the said variants hybridize under moderately stringent conditions to the nucleic acid sequence of SEQ ID No. 4 or 5, and further provided that said variants code for a protein with cytotoxic activity on CD7-bearing cells.

12. A nucleic acid sequence which is SEQ ID No. 6 or a variant thereof, wherein the variant is defined as having one or more substitutions, insertions and/or deletions as compared to the sequence of SEQ ID No. 6, provided that the said variant hybridizes under moderately stringent conditions, as defined hereinabove, to the nucleic acid sequence of SEQ ID No. 6, and further provided that the biological activity upon CD7 bearing cells of the said fusion protein comprising the modified linker sequence is substantially equal to the said activity of the fusion protein comprising the unmodified linker sequence SEQ ID No. 6.

13. An expression vector, which comprises the nucleic acid sequence of any of claims 10-12 and one or more regulatory sequences.

14. The expression vector of claim 13, which is a plasmid.

15. A host cell, which has been transformed with the vector of claim 13 or 14.

16. The host cell of claim 15, which is an eucaryotic cell.

17. The host cell of claim 16, which is a mammalian cell, plant cell, yeast cell or an insect cell.

18. The mammalian cell of claim 17, which is a CHO, COS, HeLa, 293T, HEH or BHK cell.

19. The host cell of claim 15, which is a procaryotic cell.

20. The host cell of claim 19, which is E.coli, Bacillus subtilis, or belongs to the genus of Streptomyces or Proteus L.

21. A method for producing a substantially pure fusion protein of claims 1-9, which comprises transforming a host cell with a vector of claim 13 or 14, culturing the host cell under conditions which permit expression of the sequence by the host cell and isolating the fusion protein from the host cell.

22. A therapeutic composition, comprising a therapeutically effective amount of the fusion protein of any one of claims 1-9 and a pharmaceutically acceptable carrier.

23. The therapeutic composition of claim 22, which is a vaccine.

24. Use of the composition of claim 22 or 23 in the treatment of disorders, involving a hyperproliferation of CD7-positive cells.

25. Use of the composition of claim 22 or 23 in the treatment of CD7 positive acute T-cell and/or myeloid leukemias.

26. Use of the composition of claim 22 or 23 in the prophylaxis or treatment of Graft-versus-host disease (GvHD).

27. A method for treating disorders, which involve a hyperproliferation of CD7-positive cells, in an animal, comprising:
administering to an animal in need of such treatment, a fusion protein of any of claims 1 – 9 or a composition of claim 22 or 23 in a dosage effective to substantially eliminate the hyperproliferating CD7-positive cells in said animal.

28. A method for the prophylaxis or treatment of T-cell mediated diseases or conditions of the immune system comprising administering to an animal in need thereof a T-cell depleting effective amount of a fusion protein of any of claims 1 – 9 or a therapeutic composition of claims 22 or 23 in a dosage effective to substantially deplete the T-cells in said animal.

29. A method of conditioning an animal to be transplanted with cells, or a tissue or organ of a donor, the method comprising: (a) depleting the CD7-bearing cell population in the animal; (b) providing a transplant comprising isolated bone marrow and/or stem-cell enriched peripheral blood cells of the donor, wherein the CD7-bearing cell population in a) and/or the transplant in b) are treated with a T-cell depleting effective amount of a fusion protein of any of claims 1-9 or a therapeutic composition according to claims 22 or 23; and (c) introducing the transplant into the animal.

30. The method of claims 27-29, wherein said animal is a mammal.

31. The method of claim 30, wherein said mammal is a human.

32. The method of claims 27-31, wherein the fusion protein or the composition is administered parenterally.

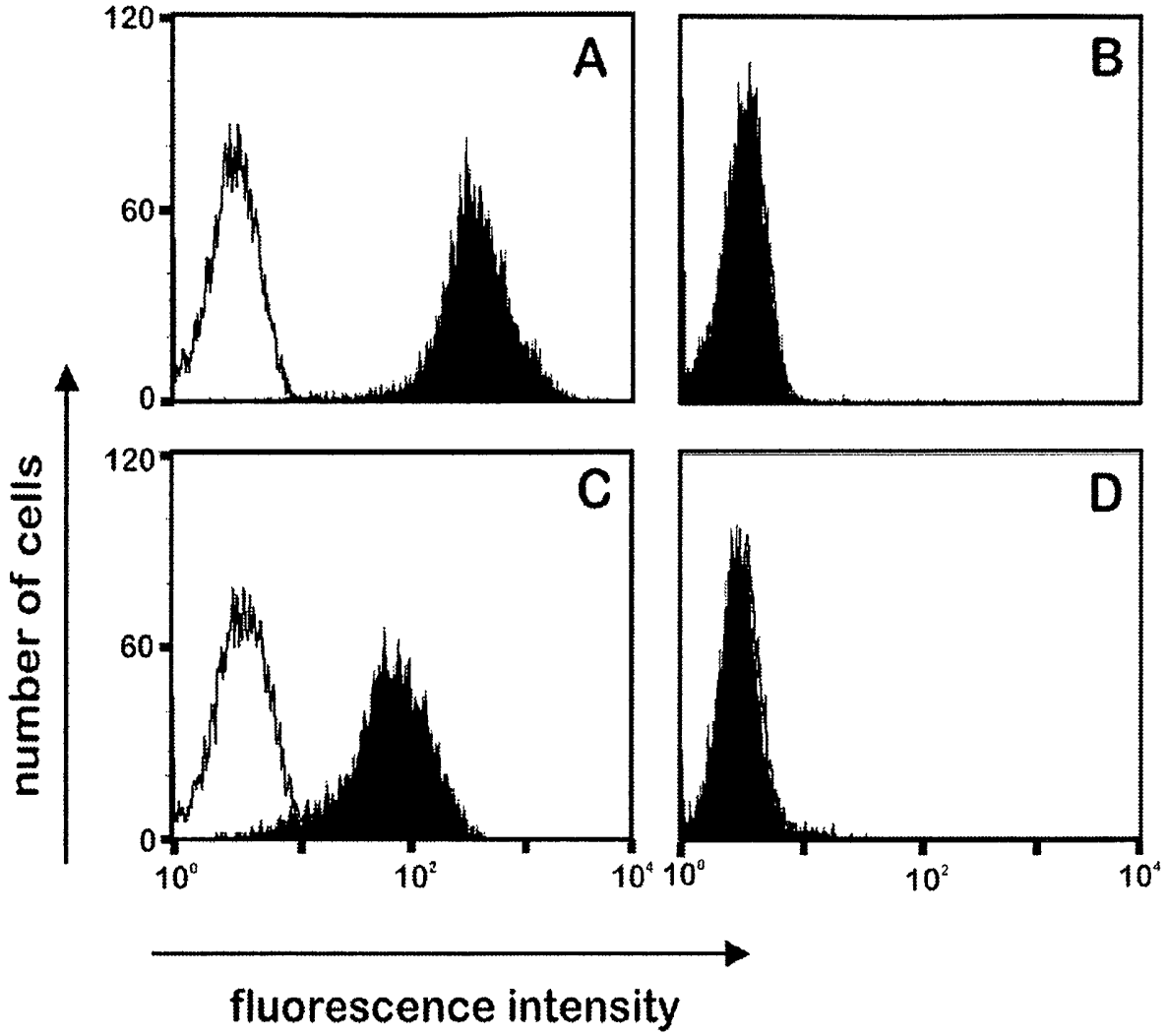


Fig. 1: The subcloned scFv fragments against CD7 and MCSP specifically bind to antigen-positive cells.

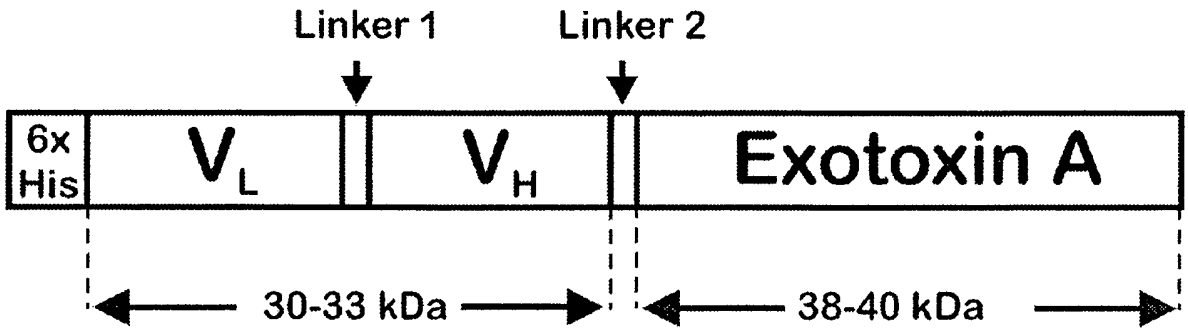


Fig. 2: Construction scheme for the recombinant immunotoxin.

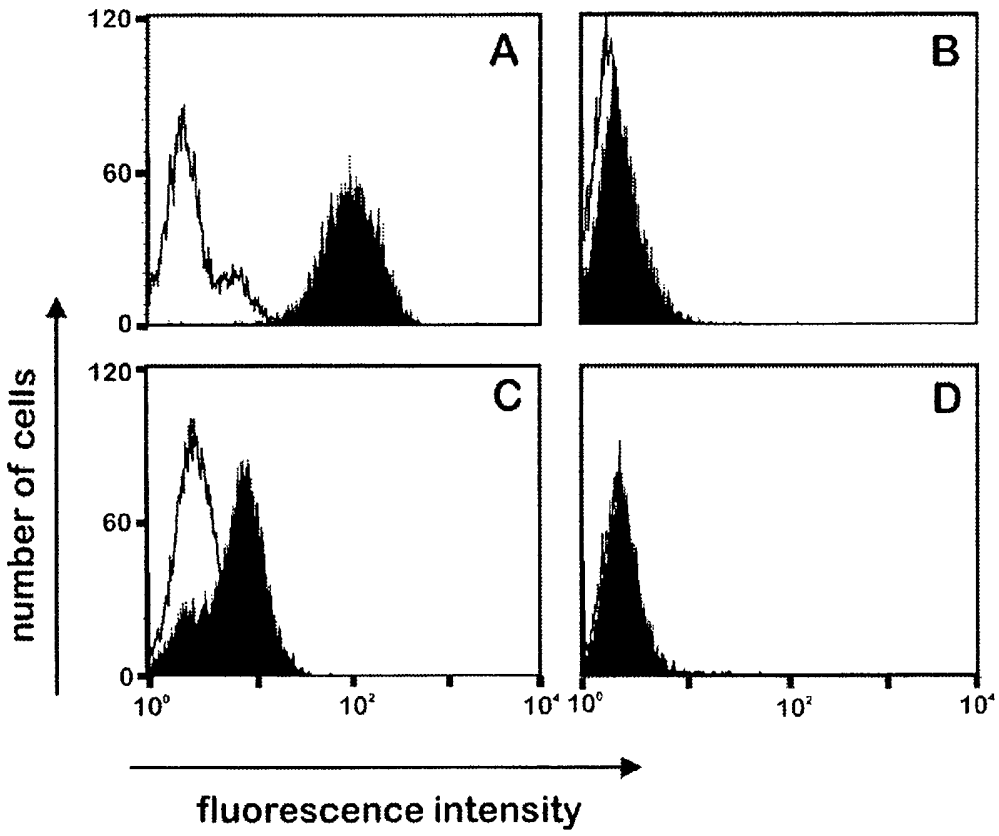


Fig. 3: ScFv fusion proteins CD7-ETA and MCSP-ETA are capable of binding to antigen-positive cells.

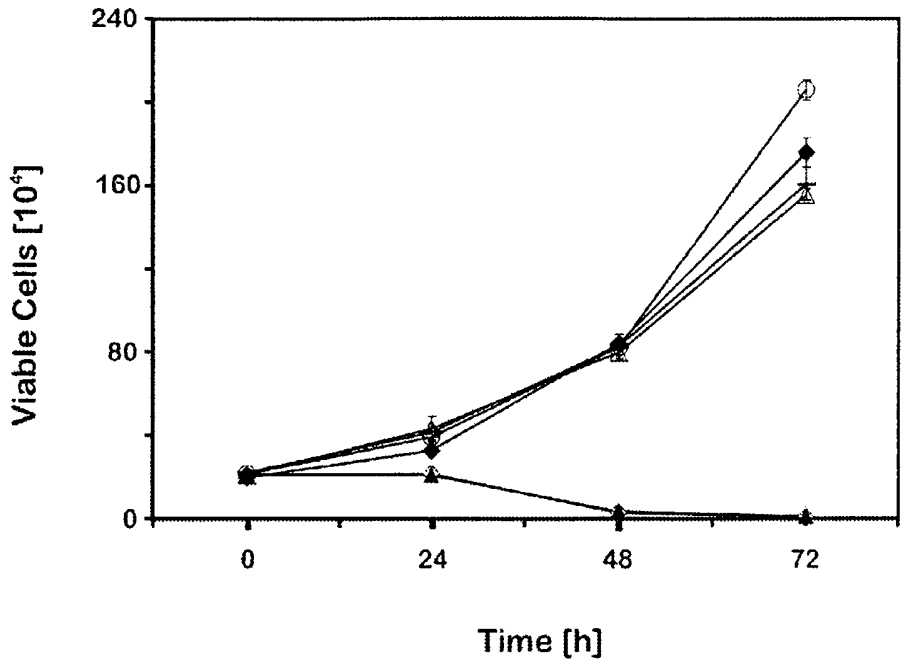


Fig. 4: Killing by CD7-ETA is blocked specifically by the parental TH-69 antibody.

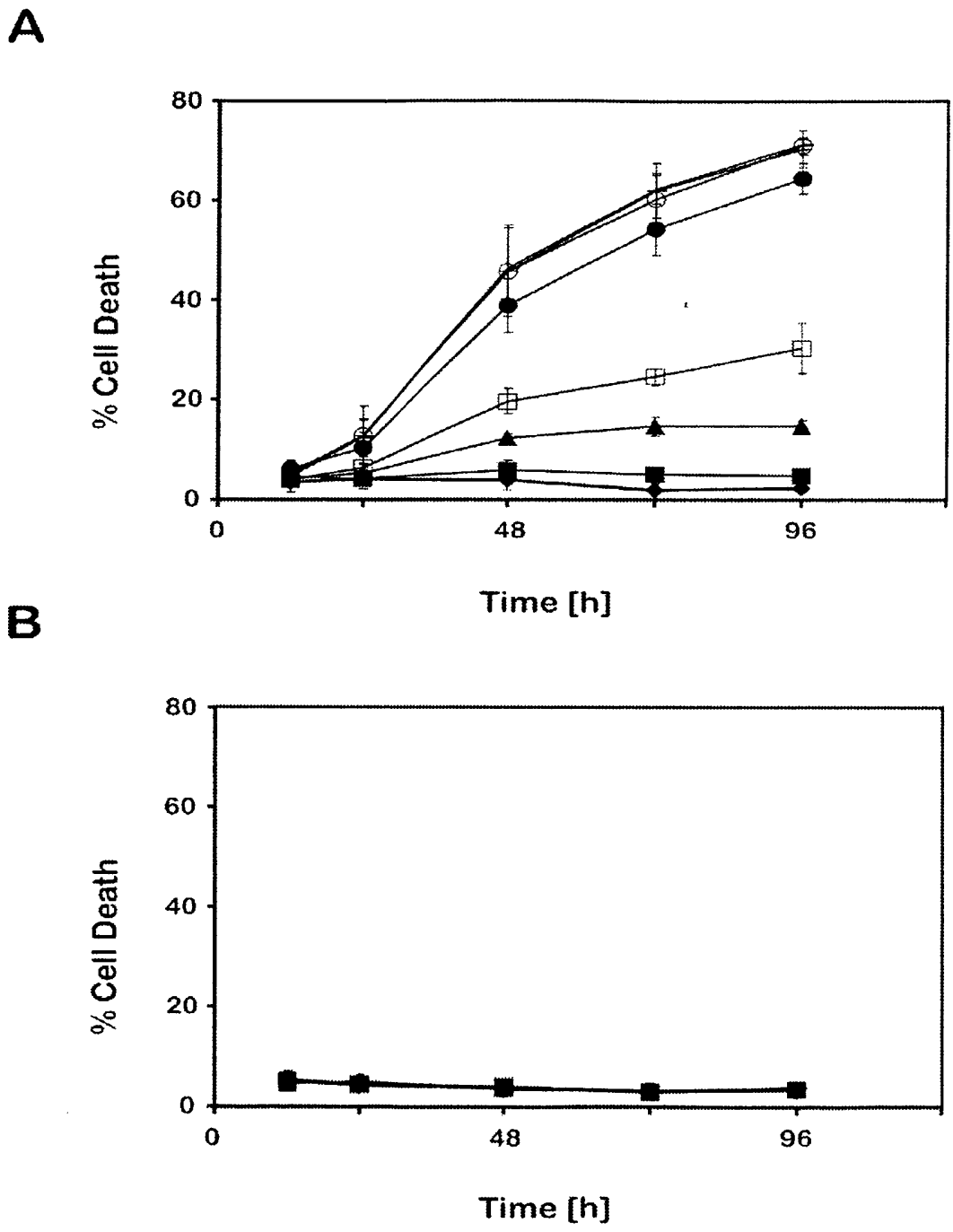


Fig. 5: CD7-ETA induces cell death of CEM leukemic blasts at low concentrations.

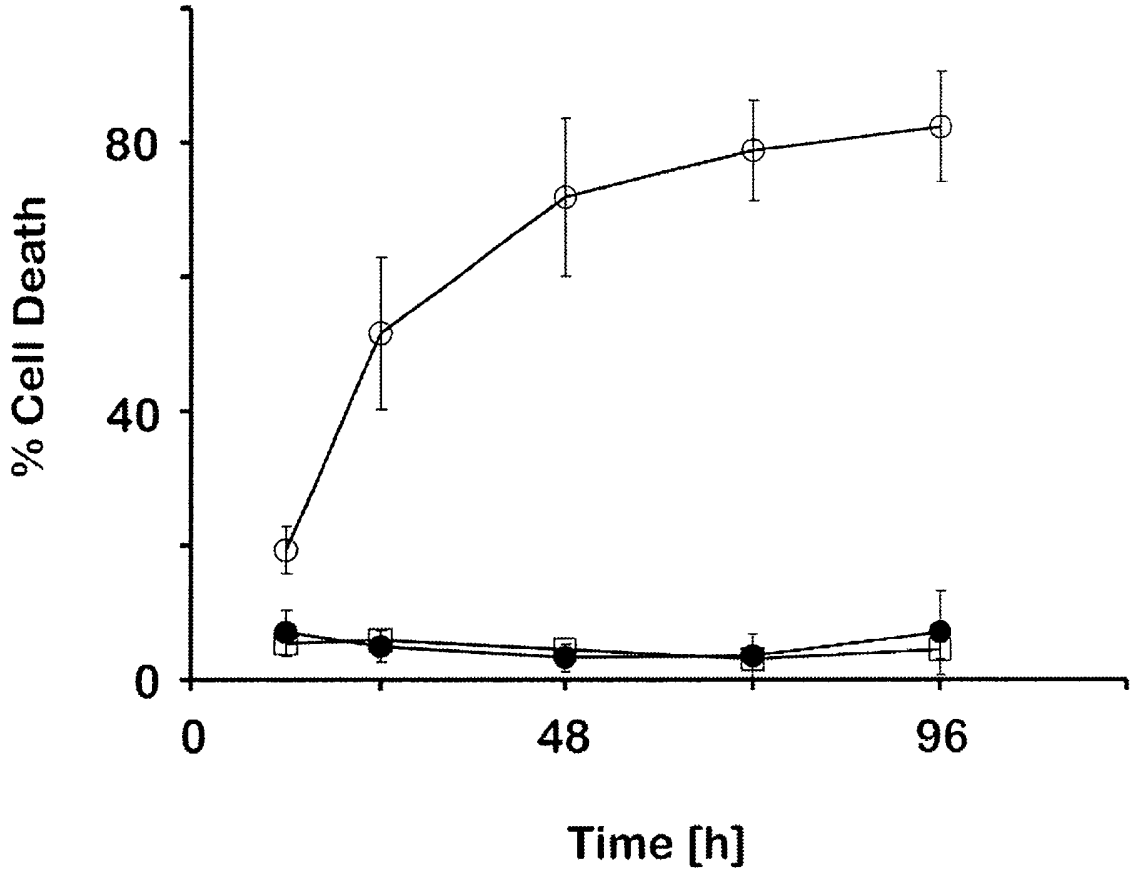


Fig. 6: CD7-ETA induces cell death of CD7-positive Jurkat T-cells but not of CD7-negative REH and NALM-6 cells.

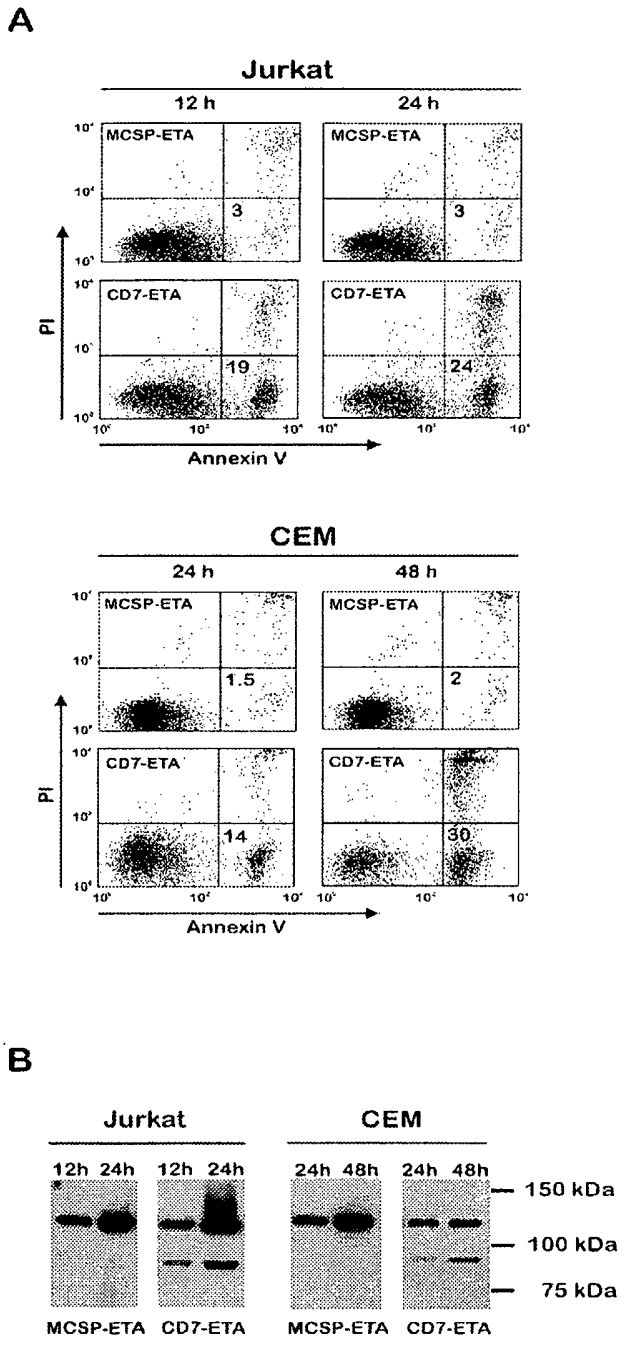
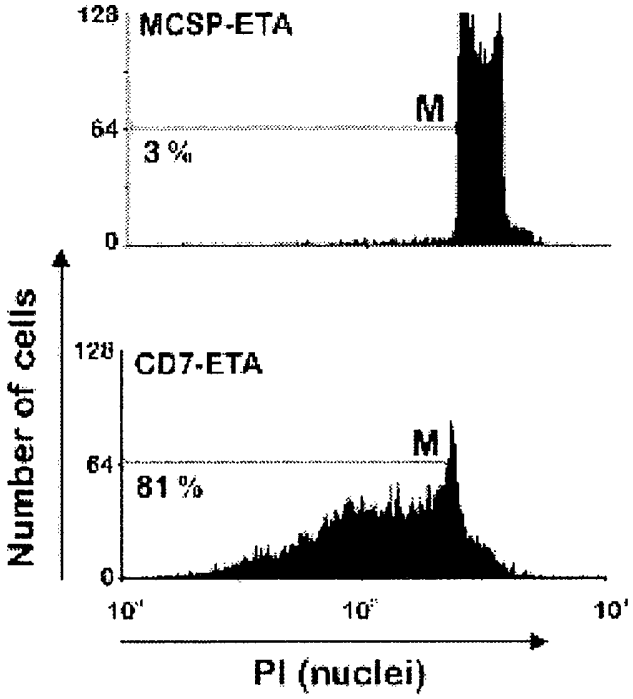


Fig. 7: CD7-ETA induces apoptosis in CEM and Jurkat cells.

A



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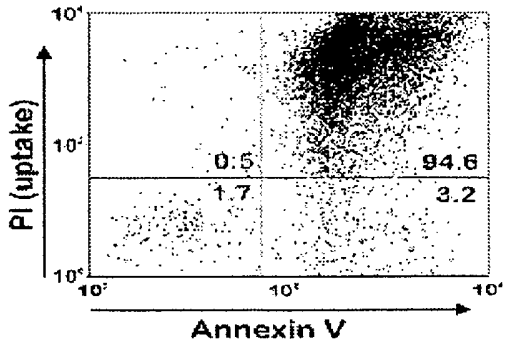

Fig. 8: Comparison of two different methods for the measurement of cell-death.

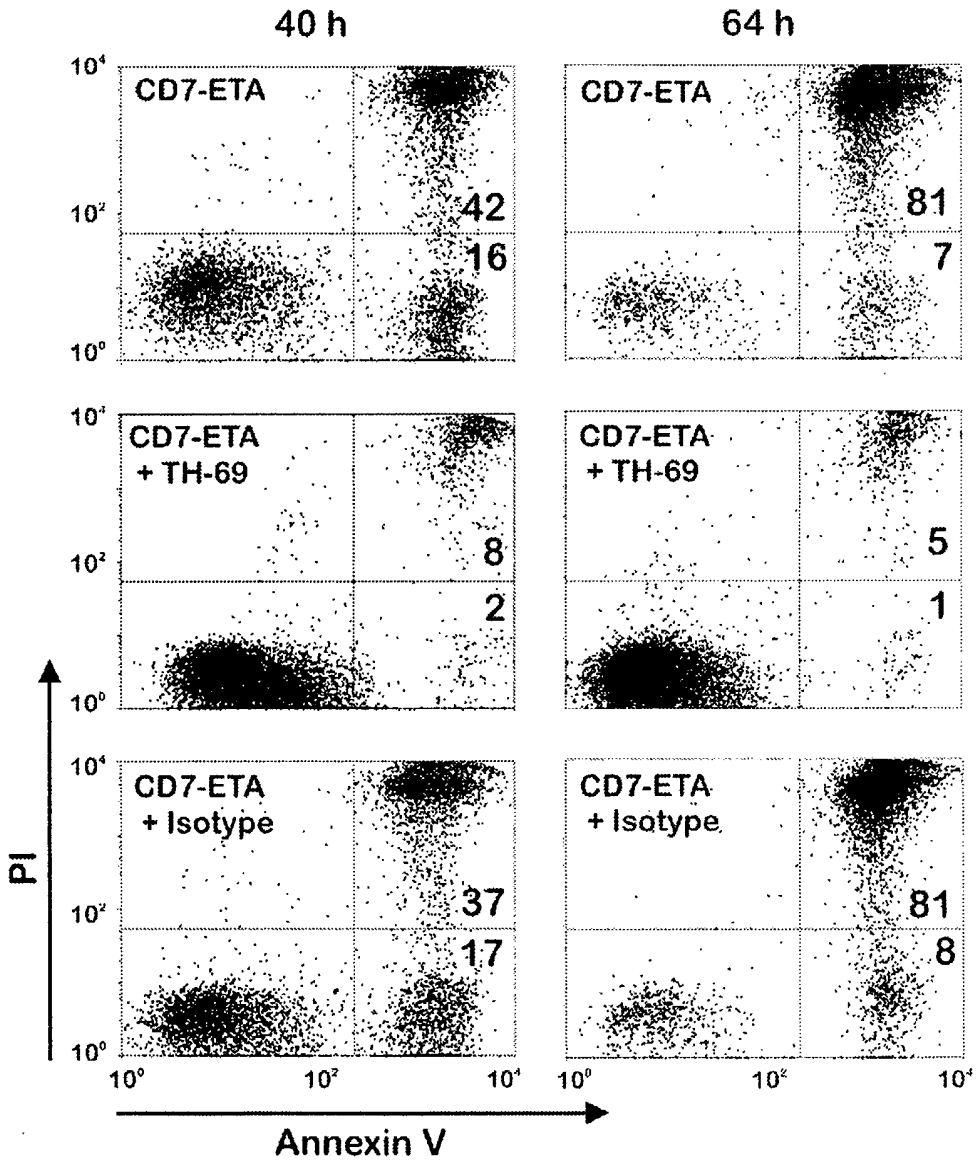

Fig. 9: Killing by the CD7-ETA scFv toxin is antigen specific as determined by competition with the parental TH-69 hybridoma antibody and appropriate controls.

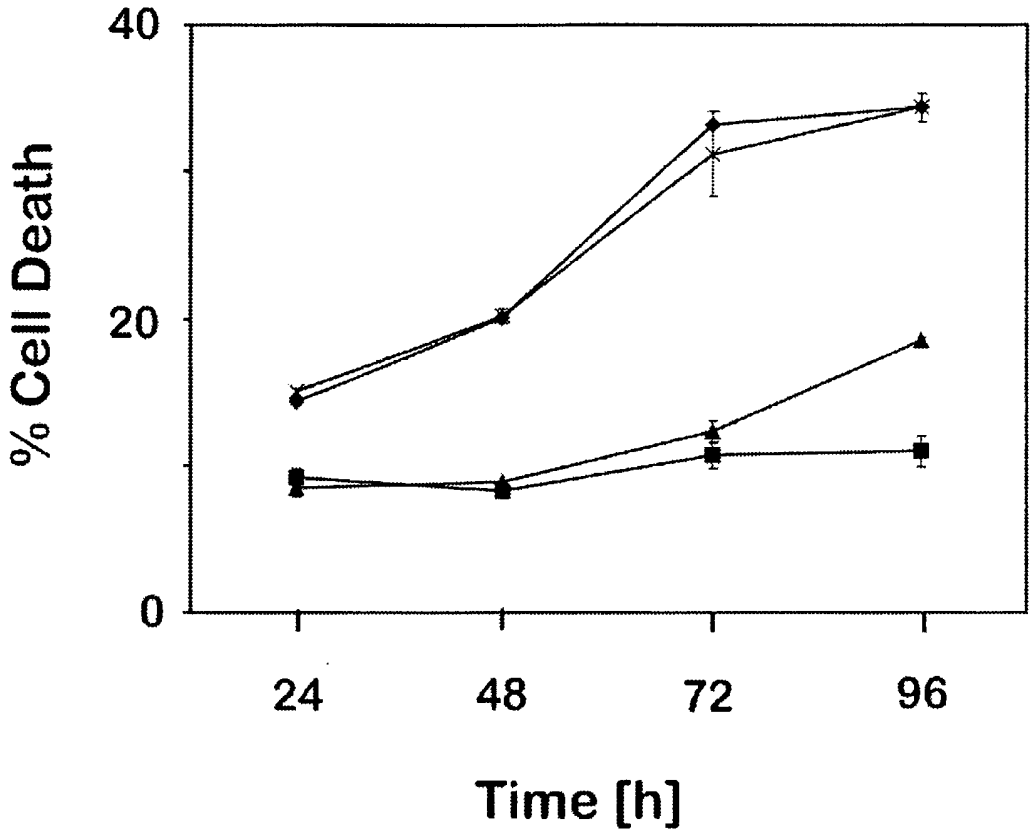

Fig. 10: Long-term primary cultures of leukemia-derived cells can be killed specifically by the CD7-ETA immunotoxin.