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Treatment of Human B Cell Lymphoma Xenografts with a CD3 × CD19 Diabody and T Cells[1]

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The use of anti-CD3 × antitumor bispecific Abs is an attractive and highly specific approach in cancer therapy. Recombinant Ab technology now provides powerful tools to enhance the potency of such immunotherapeutic constructs. We designed a heterodimeric diabody specific for human CD19 on B cells and CD3ε chain of the TCR complex. After production in *Escherichia coli* and purification, we analyzed its affinity, stability, and pharmacokinetics, and tested its capacity to stimulate T cell proliferation and mediate in vitro lysis of CD19$^+$ tumor cells. The effect of the diabody on tumor growth was investigated in an in vivo model using immunodeficient mice bearing a human B cell lymphoma. The CD3 × CD19 diabody specifically interacted with both CD3- and CD19-positive cells, was able to stimulate T cell proliferation in the presence of tumor cells, and induced the lysis of CD19$^+$ cells in the presence of activated human PBL. The lytic potential of the diabody was enhanced in the presence of an anti-CD28 mAb. In vivo experiments indicated a higher stability and longer blood retention of diabodies compared with single chain Fv fragments. Treatment of immunodeficient mice bearing B lymphoma xenografts with the diabody and preactivated human PBL efficiently inhibited tumor growth. The survival time was further prolonged by including the anti-CD28 mAb. The CD3 × CD19 diabody is a powerful tool that should facilitate the immunotherapy of minimal residual disease in patients with B cell leukemias and malignant lymphomas. ***The Journal of Immunology*****, 2000, 165: 888–895.**

Antibodies are capable of highly specific interactions with a wide variety of ligands, including tumor-associated markers and lymphocyte cell surface glycoproteins. bispecific Abs (BsAbs)[4] have significant potential for human therapy as a means of retargeting cytotoxic effector cells against tumor cells (1). CTL, for example, can be recruited for killing tumor cells if they are activated by a BsAb that binds both to the CD3 Ag associated with the TCR complex and to the target cell. Clinical studies showed tumor regression in patients treated with anti-CD3 × antitumor BsAb (2, 3). One of the best targets for BsAbs on malignant human B cells is CD19 (4). This Ag is expressed on virtually all B-lineage malignancies from acute lymphoblastic leukemia to non-Hodgkin's lymphoma. Moreover, it is not shed and is absent from hemopoietic stem cells, plasma cells, T cells, and other tissues. A possible disadvantage lies in the fact that normal B cells may also be eliminated by CD3 × CD19 BsAb treatment. However, these cells are rapidly replaced by the differentiation of new cells from the stem cell pool.

To date, BsAbs have mainly been produced using murine hybrid hybridomas (5) or by chemical cross-linking (6, 7). However, the immunogenicity of BsAb derived from rodent mAbs is a major drawback for clinical use (8). They are also difficult to produce and purify in large quantities. Recent advances in recombinant Ab technology have provided several alternative methods for constructing and producing BsAb molecules (9, 10). For example, scFv fragments have been genetically fused with adhesive polypeptides (11) or protein domains (12) to facilitate the formation of heterodimers. The genetic engineering of scFv-scFv tandems linked with a third polypeptide linker has also been conducted in several laboratories (13, 14). An alternative BsAb fragment is the scFv heterodimer diabody (15). It is formed by the noncovalent association of two single chain fusion products consisting of the V_H domain from one Ab connected by a short linker to the V_L domain of another Ab (16, 17). The two Ag binding domains have been shown by crystallographic analysis to be on opposite sides of the diabody such that they are able to cross-link two cells (18).

We recently described the construction of a bispecific diabody with dual specificity for both the human B cell Ag CD19 and ε-chain of the CD3/TCR complex designed for the treatment of minimal residual disease in patients with leukemias and malignant lymphomas (19). In this study, we detail the binding characteristics of CD3 × CD19 diabody and examine pharmacokinetics of this molecule and its in vitro and in vivo antitumor properties.

Materials and Methods

Bacterial expression constructs

For all cloning steps and DNA isolation, the *Escherichia coli* K12 strain XL1-Blue (Stratagene, La Jolla, CA) was used. Plasmids pHOG3-19 and pHOG19-3 encoding the hybrid V_H3-V_L19 and V_H19-V_L3 scFvs (19) were used as a source of genetic information to construct the improved vector for expression of bispecific CD3 × CD19 diabody. The gene coding for scFv3-19 was amplified by PCR with the primers 5-NDE, 5′-GATATA CATATGAAATACCTATTGCCTACGGC, and 3-AFL, 5′-CGAATTCT TAAGTTAGCACAGGCCTCTAGAGACACACAGATCTTTAG. The resulting PCR fragment was digested with *Nde*I and *Afl*II and cloned into the *Nde*I/*Afl*II-linearized plasmid pHKK (20) (kind gift of Dr. D. Riesenberg,

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[4] Abbreviations used in this paper: BsAb, bispecific Ab; AUC, area under curve; CLL, chronic lymphatic leukemia; RAG, recombination-activating gene; rbs, ribosome binding site; scFv, single-chain variable fragment of Ab.

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Hans-Knoll-Institute, Jena, Germany) generating the vector pHKK3-19. The gene encoding the second hybrid scFv19-3 was amplified by PCR with the primers 5-BGL, 5′-GCACACAGATCTGAGAAGGAGATATACATATGAAATACCTATTGCCTACGGC, and fe-2, 5′-CGAATTCTTAAGCTATTAGTGATGGTGATGGTGATGTGAG. The resulting PCR fragment was digested with *Bgl*II and *Afl*II and cloned into the *Bgl*II/*Afl*II-linearized plasmid pHKK3-19 generating the expression vector pHKID3 × 19. To introduce a gene encoding the Skp/OmpH periplasmic factor for higher recombinant Ab production (21), the *skp* gene was amplified by PCR with primers skp-3, 5′-CGAATTCTTAAGAAGGAGATATACATATGAAAAAGTGGTTATTAGCTGCAGG and skp-4, 5′-CGAATTCTCGAGCATTATTTAACCTGTTTCAGTACGTCGG, using as a template the plasmid pGAH317 (22), kindly provided by Dr. R. Chen (Max-Plank Institute for Biology, Tübingen, Germany). The resulting PCR fragment was digested with *Afl*II and *Xho*I and cloned into the *Afl*II/*Xho*I-digested plasmid pHKID3 × 19, resulting in the expression plasmid pSKID2. This vector contains the *hok/sok* plasmid-free cell suicide system (23) and a tetra-cistronic operon for high level production of bispecific heterodimer diabodies. The expression cassette is under the transcriptional control of the wild-type *lac* promoter/operator system and includes a short sequence coding for the N-terminal peptide of β-galactosidase (*lacZ′*) with a first rbs derived from the *E. coli lac*Z gene, followed by genes encoding the two-hybrid scFvs and Skp/OmpH periplasmic factor under the translational control of strong rbs from gene 10 of phage T7 (T7g10) (Fig. 1). The composition of the constructed plasmids was confirmed by restriction digests and dideoxynucleotide sequencing (24).

Diabody expression and purification

For functional expression of the bispecific diabody in the bacterial periplasm, the plasmid pSKID2 was transformed into *E. coli* K12 strain RV308 (Δ*lac*χ74*gal*ISII::OP308*strA*) (25), kindly provided by Dr. A. Plückthun (University of Zürich, Zürich, Switzerland). Transformed bacteria were grown overnight in shake flasks containing 2YT medium with 0.1 g/L ampicillin and 100 mM glucose ($2YT_{GA}$) at 26°C. Dilutions (1/50) of the overnight cultures in $2YT_{GA}$ were grown as flask cultures at 26°C with shaking at 200 rpm. At OD_{600} = 0.7, bacteria were harvested by centrifugation and resuspended in the same volume of YTBS medium (2YT containing 1 M sorbitol and 2.5 mM glycine betaine (26)). Isopropyl β-D-thiogalactoside was added to a final concentration of 0.2 mM, and growth was continued at 23°C for 13 h. The bacterial cells were then harvested by centrifugation, and periplasmic extracts were isolated as previously described (27, 28). Purification was achieved by immobilized copper metal affinity chromatography (IMAC) on Cu^{2+}-charged chelating Sepharose (Amersham Pharmacia Biotech, Freiburg, Germany), as previously described (28). The final purification of the CD3 × CD19 diabody was achieved by ion-exchange chromatography on a Mono S HR5/5 column (Pharmacia) in 50 mM imidazole-HCl, pH 6.4, with a linear 0–1 M NaCl gradient. The purified diabody preparations were dialyzed against PBSI buffer (15 mM sodium phosphate, 0.15 M NaCl, 50 mM imidazole, pH 7). All purification procedures were performed at 4°C. Isolation of the anti-CD19 and anti-CD3 scFv fragments was performed as previously described (28, 29).

Flow cytometry

The human $CD3^+/CD19^-$ acute T cell leukemia line Jurkat and the $CD19^+/CD3^-$ B cell line JOK-1 were used for flow cytometry, as previously described (19). In brief, 5×10^5 cells in 50 μl RPMI 1640 medium (Life Technologies, Eggestein, Germany) supplemented with 10% FCS and 0.1% sodium azide (referred to as complete medium) were incubated with 100 μl of a recombinant Ab preparation for 45 min on ice. After washing with complete medium, the cells were incubated with 100 μl of 10 μg/ml anti c-*myc* mAb 9E10 (IC Chemikalien) in the same buffer for 45 min on ice. After a second washing cycle, the cells were incubated with 100 μl of FITC-labeled goat anti-mouse IgG (Life Technologies) under the same conditions as before. The cells were then washed again and resuspended in 100 μl of 1 μg/ml solution of propidium iodide (Sigma, Deisenhofen, Germany) in complete medium to exclude dead cells. The relative fluorescence of stained cells was measured using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Affinity determination

The apparent affinities of the bispecific diabody were determined from competitive inhibition assays with FITC-labeled mAb OKT3 (anti-CD3) and HD37 (anti-CD19), as previously described (19, 28). Binding affinities were calculated according to the following equation (30): $K_{d(I)} = IC_{50}/(1 + (\text{FITC-mAb})/K_{d(mAb)})$, in which I is the unlabeled inhibitor (diabody), FITC-mAb is the concentration of FITC-labeled mAb, $K_{d(mAb)}$ is the binding affinity of mAb, and IC_{50} is the concentration of inhibitor that yields 50% inhibition of binding. Affinity constant (K_d) values of 0.8 and 0.4 nM were assigned to mAb OKT3 (31) and HD37 (19), respectively.

In vitro cell surface retention

Cell surface retention assays were performed at 37°C under conditions preventing internalization of cell surface Ags, as described (32), except that the detection of the retained diabody was performed using anti-c-*myc* mAb 9E10 (IC Chemikalien), followed by FITC-labeled anti-mouse IgG. Kinetic dissociation constants (k_{off}) were calculated using a first order equation $F_t = F_0 \times e^{-kt}$, in which F_t is fluorescence at time t, F_0 is fluorescence at time 0, and k is k_{off}. The $t_{1/2}$ for dissociation of diabody was calculated from the equation $t_{1/2} = \ln 2/k_{off}$.

Cell proliferation assay

Human PBL (1×10^4/well) were cultured together with irradiated (120 rad) $CD19^+$ Burkitt lymphoma Raji cells (2.5×10^5/well) in 96-well plate for 5 days in IMEM (Sigma) supplemented with 10% human AB serum (Sigma), 25 IU/ml of human rIL-2 (Chiron, Emeryville, CA), and varying amounts of diabody. [^{3}H]Thymidine (0.5 μCi/well) was added 12 h before harvesting. The cells were harvested, and [^{3}H]thymidine incorporation was measured with a liquid scintillation beta counter (Beckman, Palo Alto, CA).

In vitro cytotoxicity

The efficacy of the diabody in mediating tumor cell lysis by activated human PBLs was determined using the JAM test (33) or chromium release assay. For the JAM test, 10^5 activated human PBLs prepared as previously described (19) were mixed in round-bottom microtiter plates with 1×10^4 of target cells (Raji or tumor cells from a CLL patient) labeled with [^{3}H]thymidine (E:T ratio = 10:1) in 100 μl medium plus 50 μl of Ab sample. After incubating the plate at 37°C, 5% CO_2 for 4 h, the cells were harvested and radioactivity was measured with a liquid scintillation beta counter. To analyze the specificity of diabody-mediated tumor cell lysis, in some experiments either mAb OKT3 (anti-CD3) or mAb HD37 (anti-CD19) was added at concentrations up to 100 μg/ml. Chromium release assays using ^{51}Cr-labeled Raji cells were performed essentially as previously described (19).

Analysis of diabody stability in vitro

The diabody preparation was diluted (at least 20-fold) in human serum (Sigma) to a concentration of 15 μg/ml and sterilized by filtration through a Membrex 4CA filter with a void volume of 50 μl and a pore size 0.2 μm (MembraPure, Lörzweiler, Germany). One-hundred-microliter aliquots were stored under sterile conditions at 37°C. At given time points, the aliquots were frozen and kept at −80°C. Activities of the samples after storage were determined by flow cytometry using $CD3^+$ Jurkat cells. Protein degradation in each sample was determined by 12% SDS-PAGE, followed by Western blot analysis using anti-c-*myc* mAb 9E10, as previously described (34).

Radioiodination

Diabody or scFv solutions (1 mg/ml in 50 mM Tris-HCl, 1 M NaCl, pH 7) were placed in glass tubes coated with 20 μg of IODO-Gen (Pierce, Rockford, IL) per mg of protein. After adding 3 mCi of $Na^{125}I$ (Amersham Buchler, Braunschweig, Germany), the resulting mixture was incubated for 10 min at room temperature. Unincorporated radioiodine was separated from the labeled protein by size-exclusion chromatography using Bio-Gel P6 (Bio-Rad, Munich, Germany) and PBSI as the elution buffer. The final sp. act. of anti-human CD3 scFv and the CD3 × CD19 diabody were 2.4 and 6.6 mCi/mg, respectively.

Pharmacokinetic studies

Male NMRI mice, each weighting ~40 g (24–27 animals for each labeled protein), were injected into the tail vein with 200 μl of PBSI containing 10 μg of human serum albumin and 5 μg of labeled Ab fragments. At the indicated time points, animals in triplicates were anesthetized, bled, and sacrificed in accordance with local animal protection laws. For scFv, the blood samples were obtained at 5, 10, 20, 40, 90, 180, 360, and 1080 min after injection. The time points for the diabody were the same except that the last animal groups were sacrificed 24 h after injection of the labeled compound. Blood samples were counted using a gamma counter. Blood content was corrected for protein-bound radioactivity (see below) and expressed as a percentage of injected dose per gram of blood (%ID/g). AUC was calculated using GraphPad Prism (GraphPad Software, San Diego, CA) and expressed as %ID/g × min. The $t_{1/2}\alpha$ was defined by time points

5, 10, 20, and 40 min, while the $t_{1/2}\beta$ was defined by time points from 1.5 to 24 h.

Analysis of diabody stability in vivo

Two hundred microliters of blood from each sacrificed animal were mixed with 10 μl of heparin (5000 IU/ml; Braun Melsungen, Melsungen, Germany), followed by sedimentation of cellular material and TCA precipitation of the supernatant. Radioactivity associated with pellets and supernatants was counted and expressed as a percentage of total radioactivity (cpm) for each specific time point. Pooled plasma samples from the earlier time points (up to 1.5 h) were also analyzed by 12% SDS-PAGE, followed by autoradiography.

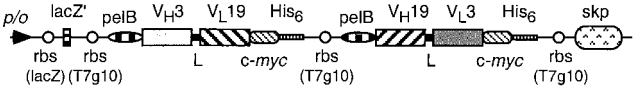
Treatment of human B cell lymphoma in RAG2-deficient mice

All animal experiments were performed in accordance with the guidelines of the German Cancer Research Center and the animal-protection laws of the Bundesland Baden-Württenberg and of the Federal Republic of Germany. RAG2-deficient mice were bred and kept under specific pathogen-free conditions at the Central Animal Facilities of the German Cancer Research Center. In each experiment, cohorts of five animals were used to permit accurate comparison among differently treated groups. Mice were given s.c. 10^8 Raji cells. To reduce inherent NK cell activity, the mice were irradiated (300 rad) 1 day before tumor inoculation and received i.p. injections of 25 μg mAb PK136 (anti-IL-2R) all 5 days during the whole experiment. At days 5, 12, and 20 after tumor inoculation, the animals received i.v. 5×10^6 human PBLs that were preactivated in vitro by immobilized mAb OKT3 (anti-CD3), soluble mAb 15E8 (anti-CD28), and low-dose IL-2. A few hours after each PBL injection, either PBS or 50 μg of the bispecific diabody, or the quadroma-derived BsAb OKT3 $\times$ HD37 (35), or combinations of these with 50 μg of anti-CD28 mAb 15E8, respectively, were administered i.v. Tumor size was measured using a caliper every second day. The mean diameter and SE of each group were calculated. In addition, days of sacrifice were recorded and used for estimation of survival time. Animals were followed until the s.c. tumors reached a maximal tolerated size of 15 mm in diameter and were sacrificed by cervical dislocation. The data on tumor growth were evaluated by a linear mixed-effects model fit by REML (restricted maximum likelihood) (36, 37) using the software S-Plus 3.4 (Sun SPARC station). The *p* values were calculated for intercepts on the *y*-axis (*p1*) and for the slopes of tumor growth curves (*p2*). Therefore, *p1* indicates the significance of the assumed start of tumor growth, and *p2* the significance of differences in tumor growth rates. The *p* values for differences in survival times were calculated using the log rank test. The results of a statistical test were assumed to be significant if the *p* value was less than 5%.

Results

Expression and purification of CD3 $\times$ CD19 diabody

To obtain a BsAb suitable for the therapy of human B cell malignancies, we constructed a small recombinant molecule with dual specificity for both the human B cell surface Ag CD19 and the signal-transducing CD3ϵ chain of the human TCR/CD3 complex. ScFv fragments derived from the hybridomas HD37 (38) and OKT3 (39) were used to create a bispecific CD3 $\times$ CD19 diabody (19). To increase the yield of functional heterodimer (diabody) formed in the bacterial periplasm by cosecretion of the two hybrid scFvs V_H3-V_L19 and V_H19-V_L3, we constructed an optimized expression vector pSKID2 (Fig. 1). The vector contains several features that improve plasmid performance and lead to increased accumulation of functional bivalent product in the *E. coli* periplasm under conditions of both shake-flask cultivation and high cell density fermentation. These are the *hok/sok* postsegregation killing system (23), which prevents plasmid loss, strong tandem rbs (20), and a gene encoding the periplasmic factor Skp/OmpH that increases the functional yield of Ab fragments in bacteria (21). The use of an improved expression system allowed us to isolate a functional bispecific diabody with a yield of 10 mg per 1 l of shake-flask culture and a purity greater than 95% (data not shown). The size-exclusion chromatography on a calibrated Superdex 200 column revealed a predominantly dimeric form of the diabody with only a small proportion of putative tetramers (data not shown).



L = akttpKLGG

FIGURE 1. Schematic representation of tetra-cistronic operon encoding the bispecific anti-human CD3 $\times$ CD19 diabody in plasmid pSKID2. The locations of wild-type *lac* promoter/operator (*p/o*), rbs either from *E. coli lac*Z gene (lacZ) or from gene 10 of bacteriophage T7 (T7g10), gene encoding the N-terminal peptide of β-galactosidase (lacZ′), c-*myc* epitope (c-*myc*), hexahistidine tag (His_6), and *skp*/*omp*H gene (*skp*) are indicated. Amino acid sequence of the linker (L) connecting V domains in each hybrid scFv is shown below the drawing. In the linker, small letters indicate amino acids derived from the C_H1 domain. The residues introduced artificially during the cloning scFv genes (19) are shown in block letters.

Ag-binding affinity

Flow cytometry experiments demonstrated a specific interaction of the diabody with both human $CD19^+$ JOK-1 and $CD3^+$ Jurkat cells. However, the fluorescence intensities obtained for interaction with JOK-1 cells were significantly higher than for Jurkat cells, reflecting the difference in affinity values for the two Ag binding sites (Fig. 2). The CD19- and CD3-binding affinities of the diabody were estimated by competitive binding to human JOK-1 and Jurkat cells in the presence of either FITC-labeled mAb HD37 (anti-CD19) or OKT3 (anti-CD3). The relative affinities were calculated from the corresponding IC_{50} values (19). The affinity of the diabody for $CD19^+$ target B cells was 10-fold higher than its affinity for $CD3^+$ effector T cells (Table I). Theoretically, the cosecretion of two hybrid scFv fragments may give rise to two types of dimer: active heterodimers and homodimers that are probably inactive. However, affinity measurements indicated that the diabody was mostly, if not completely, in the active heterodimeric form. It bound to both human CD3 and CD19 with affinities comparable with that of the parental scFv fragments (19). To investigate the biological relevance of the differences in affinity values for different Ags, the in vitro retention of the diabody on the surface of both $CD3^+$ and $CD19^+$ cells at 37°C was determined by flow cytometry (Fig. 3). The diabody had a relatively short retention $t_{1/2}$ on $CD3^+$ Jurkat cells (3 min) and 3-fold longer $t_{1/2}$ on the surface of $CD19^+$ JOK-1 cells, reflecting the lower CD3-binding affinity deduced from inhibition experiments (Table I).

Biological activity of CD3 $\times$ CD19 diabody

Three different in vitro tests were used for analyzing the biological activity of the bispecific diabody. First, its ability to stimulate T cell proliferation when bound to the surface of tumor cells was determined. Irradiated $CD19^+$ Burkitt lymphoma Raji cells were treated with different concentrations of CD3 $\times$ CD19 diabody and subsequently incubated in the presence of human PBL prestimulated with a low dose of IL-2. In this assay, we observed a clear dose-dependent increase of effector cell proliferation (data not shown). The ability of the bispecific diabody to induce tumor cell lysis by redirecting T cell-mediated cytotoxicity was investigated using a JAM test that is based on measuring DNA fragmentation in the target cell as a result of apoptosis (33). The death of $CD19^+$ Raji cells was specifically triggered by the diabody in a concentration-dependent manner using an E:T ratio of 10:1 (Fig. 4*A*). Even with a rather low E:T ratio of 5:1, we observed a fairly high specific Raji cell killing of 46.4% with a diabody concentration of 10 μg/ml (data not shown). A time-course measurement revealed that the maximal plateau of tumor cell lysis was reached after 4–5

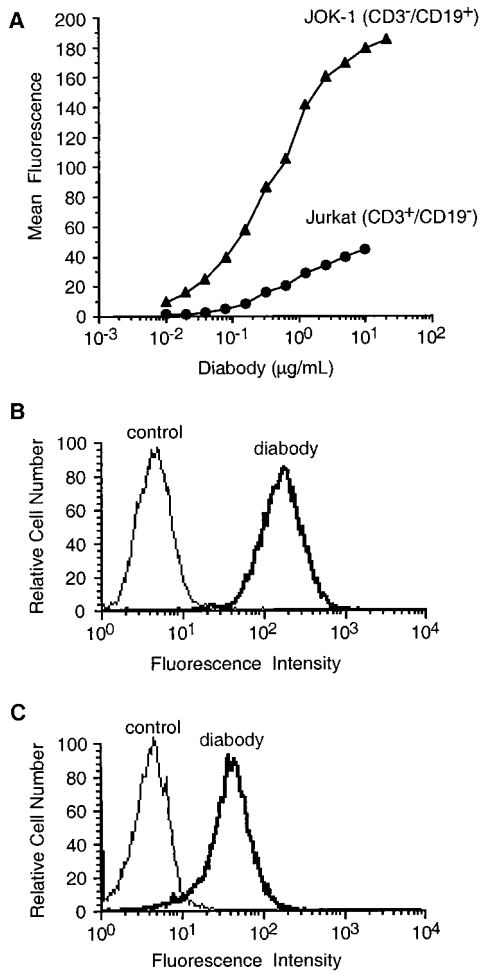


FIGURE 2. Flow-cytometric analysis of diabody binding to $CD19^+/CD3^-$ JOK-1 cells and $CD3^+/CD19^-$ Jurkat cells. *A*, Shows fluorescence dependence on diabody concentration. Results of diabody binding at concentration 10 μg/ml to either JOK-1 or Jurkat cell are shown in *B* and *C*, respectively.

h (data not shown). The specificity of diabody-mediated tumor cell lysis was demonstrated by inhibition of cytolysis in the presence of parental monospecific anti-CD3 and anti-CD19 mAbs (Fig. 4*B*). Furthermore, the CD3 $\times$ CD19 diabody was not able to mediate the lysis of $CD19^-$ Lovo cells, and a monospecific CD19 $\times$ CD19 diabody (40) was not able to mediate the lysis of $CD19^+$ Raji cells (data not shown).

To investigate the efficacy of the CD3 $\times$ CD19 diabody to target T cell-mediated lysis of authentic tumor cells, we used PBL of a patient suffering from CLL. We enriched T cells from the patient's peripheral blood by nonadherence on nylon wool. Autologous PBLs (containing over 90% $CD19^+$ leukemic cells) were used as target cells in a standard JAM test kill assay. Using an E:T ratio of 10:1, we observed a concentration-dependent increase of target

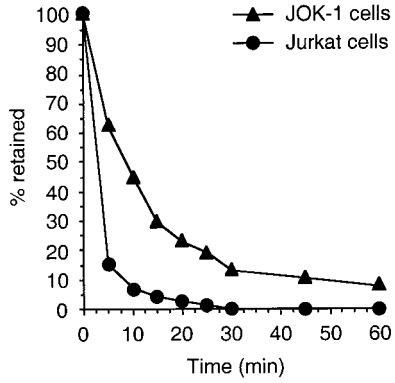


FIGURE 3. Analyses of diabody retention on the surface of either $CD19^+/CD3^-$ JOK-1 cells or $CD3^+/CD19^-$ Jurkat cells at 37°C, as determined by flow cytometry.

cell lysis from 20% at a diabody concentration of 1 μg/ml to more than 50% at a concentration of 50 μg/ml (Fig. 4*A*). Several authors have shown that T cells, redirected against tumor cells with bispecific antitumor $\times$ anti-CD3 Abs, can be effectively costimulated using anti-CD28 mAb or antitumor $\times$ anti-CD28 BsAb to increase their cytolytic activity (5, 41, 42). We therefore investigated whether the addition of anti-CD28 mAb 15E8 in a concentration of 1 μg/ml to the effector cell population could increase target cell lysis. A human B cell line Raji expressing CD19 was used as a target. The effect of the bispecific diabody was measured using a standard ^{51}Cr release assay based on plasma membrane disintegration and the consequent release of cytoplasm. A mixture of both parental scFv fragments (anti-CD3 and anti-CD19) served as a negative control for determining the background lysis. As shown in Fig. 5, the anti-CD28 mAb significantly increased the lytic capacity of redirected PBL. Moreover, the increased cytotoxicity of the diabody in the presence of mAb 15E8 was independent of the E:T ratio (Fig. 5).

Diabody stability in vitro

One of the most important applications of BsAbs is in cancer therapy in which factors such as plasma $t_{1/2}$ and tumor penetration play an important role. The BsAb should therefore be stable at 37°C in human serum to have a significant antitumor effect. One might expect that the bispecific diabody would be rather labile, as it was formed by noncovalent interactions of two hybrid scFv molecules. We therefore investigated the stability of the diabody when stored at a fairly low concentration in human serum at 37°C for prolonged periods of time. The residual activity was estimated by flow cytometry. We found that the bispecific diabody retained 80% of its Ag-binding activity after 24-h incubation at 37°C in human serum and had a $t_{1/2}$ of almost 3 days (Table II). Western blot analyses of the samples of the diabody showed no proteolytic degradation after 81 h of incubation in human serum at 37°C, indicating that the loss of activity was mainly caused by aggregation of the diabody (data not shown).

Table I. *Affinity and binding kinetics of CD3 $\times$ CD19 diabody to cell surface bound Ags*

Ag	IC_{50} (μg/ml)[a]	IC_{50} (nM)[a]	K_d (nM)[a]	k_{off} ($s^{-1}/10^{-3}$)[b]	$t_{1/2}$ (min)[b]
CD19	1.47	25.25	1.29	1.16 $\pm$ 0.28	10.0
CD3	9.00	154.58	15.46	3.97 $\pm$ 1.42	2.9

[a] Deduced from inhibition experiments.
[b] Deduced from cell surface retention assays.

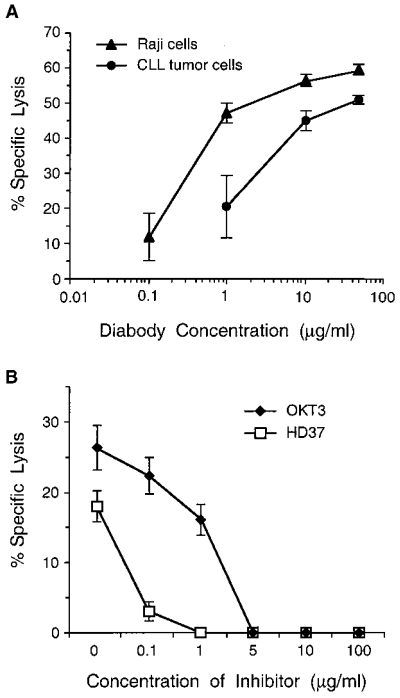


FIGURE 4. Redirected tumor cell lysis by preactivated human PBLs, as determined by a JAM test. *A*, Dose-dependent diabody-mediated lysis of either Raji cells or tumor cells from a CLL patient at the E:T ratio of 10:1. *B*, Inhibition of diabody-mediated lysis of $CD19^+$ Raji cells in presence of either anti-CD3 mAb OKT3 or anti-CD19 mAb HD37. Diabody was used at concentration 0.5 μg/ml; E:T ratio was 5:1. Mean values ± SE of three independent experiments were plotted. In all experiments, the spontaneous cell lysis did not exceed 10%.

Blood clearance and stability in vivo

To estimate the plasma clearance pharmacokinetics of the diabody in comparison with a smaller scFv fragment, we administered preparations of ^{125}I-labeled anti-human CD3 scFv monomer (30 kDa) (28) and CD3 × CD19 diabody (58 kDa) into normal mice. Blood samples were taken at various time points. To exclude errors in calculating plasma $t_{1/2}$ due to free radioiodine resulting from cellular, metabolic degradation of labeled protein, we first determined the proportion of protein-bound radioactivity for each plasma sample by TCA precipitation. The TCA-precipitable radioactivities of scFv decreased rapidly in the blood over time; only 50% of the radioactivity was bound to protein 30 min after the i.v. injection. In contrast, the diabody lost its label much more slowly;

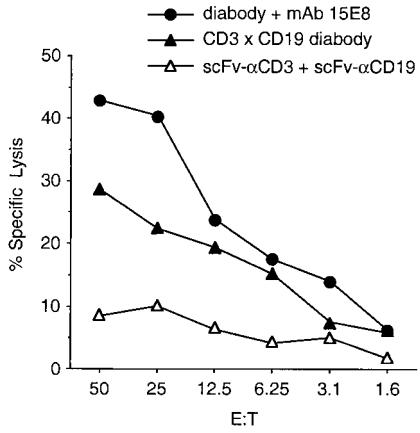


FIGURE 5. Diabody-mediated lysis of $CD19^+$ Raji cells by activated human PBL. The ^{51}Cr-labeled target cells were coincubated with effector cells at different E:T ratios for 4 h. Percent cytotoxicity was calculated based on ^{51}Cr release in the presence of 2.5 μg/ml of parental anti-CD3 and anti-CD19 scFvs, 2.5 μg/ml of diabody, or 2.5 μg/ml of diabody plus 1 μg/ml of anti-CD28 mAb 15E8.

more than 50% of its radioactivity could be precipitated 3 h after injection (data not shown). The estimated $t_{1/2}$ of the diabody was more than three times that of the scFv (Table II). Analyses of blood samples at different time points demonstrated an extremely rapid clearance of scFv from plasma, with an α-phase (extravasation and renal clearance) $t_{1/2}$ ($t_{1/2}\alpha$) of 3.5 min and a β-phase (catabolism and slow release of extravasated Ab from perivascular space back into the blood) $t_{1/2}$ ($t_{1/2}\beta$) of 74.6 min (Table II). The diabody had an ~2-fold slower first-pass clearance and a somewhat slower catabolism (Table II). The pharmacokinetic AUC were calculated for both Ab fragments to provide a means of estimating the total relative dose that would be delivered in a therapeutic application. The diabody demonstrated the best pharmacokinetic performance; its AUC value was more than 2-fold higher than AUC values calculated for the scFv (Table II). The differences in the pharmacokinetic properties of these constructs correlate quite well with their size.

Effect of diabody on xenotransplanted B cell lymphoma

To estimate the in vivo antitumor activity of the CD3 × CD19 diabody, we established a xenotransplant model of the Raji Burkitt's lymphoma in RAG2-deficient mice. Cohorts of five mice were inoculated s.c. with 10^8 tumor cells, and at days 5, 12, and 20 received i.v. 5×10^6 human PBLs preactivated in vitro by immobilized mAb OKT3 (anti-CD3), soluble mAb 15E8 (anti-CD28),

Table II. *In vitro and in vivo stability and pharmacokinetic parameters of CD3 × CD19 diabody in comparison with anti-CD3 scFv*

	Stability[a]		Pharmacokinetics[b]		
			Blood Clearance		
Ab Fragment	In vitro, $t_{1/2}$ (h)	In vivo, $t_{1/2}$ (h)	$t_{1/2}\alpha$ (min)	$t_{1/2}\beta$ (min)	AUC (% ID/g × min)
scFv	ND	1.2	3.5	74.6	422.4
Diabody	63.0	3.1	8.4	91.9	1123

[a] The half-life values for stability measurements were deduced from the one phase exponential decay fit of experimental data and were calculated as ln2/*k*.

[b] Blood content was corrected for protein bound radioactivity and expressed as a percentage of injected dose per gram of blood (% ID/g). To estimate the blood retention half-lives, a bi-exponential function was fitted to each blood clearance curve. The therapeutic effectiveness of the analyzed Ab fragments was also compared by integration of the blood retention curves. The areas under the curve (AUC) were measured to infinite time with each curve being represented as a bi-exponential function.

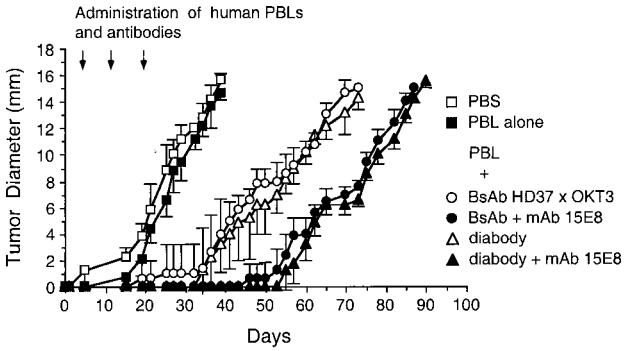


FIGURE 6. Effects of Ab therapy on the growth of Raji s.c. xenografts in RAG2 mice. All animal groups were inoculated with Raji Burkitt lymphoma on day 0. The mice received either PBS, preactivated human PBLs alone, or preactivated human PBLs, followed a few hours later by the administration of BsAb OKT3 × HD37, CD3 × CD19 diabody, BsAb OKT3 × HD37 plus anti-CD28 mAb 15E8, CD3 × CD19 diabody plus mAb 15E8. Arrows indicate the time points of administration of human PBLs (days 5, 12, and 20). Means of each group and SE are plotted.

and low-dose IL-2. A few hours after each PBL inoculation, the mice were treated either with no Ab, the bispecific diabody, or BsAb OKT3 × HD37 (35) administered as a tail vein injection. Additional groups received BsAb in combination with anti-CD28 mAb 15E8. All the animals in the control groups receiving only PBS or PBL developed tumors larger than 1 cm in diameter (0.4–0.6 cm^3) within 4 wk (Fig. 6). In contrast, the BsAb OKT3 × HD37 and diabody demonstrated tumor growth suppression till days 19 and 34, respectively (Fig. 6). Administration of both BsAb and diabody in combination with anti-CD28 mAb further prolonged the period of tumor suppression till days 46 and 55, respectively (Fig. 6). After reaching a certain size, the tumors grew progressively in all animal groups. There was no significant difference in tumor growth between two control groups receiving either PBS or activated PBL alone ($p1 = 0.0688$, $p2 = 0.0696$; Fig. 6), indicating that under the conditions used, any allogeneic reaction of the effector cells toward the tumor can be ignored. There was also no significant difference in tumor growth between the animals receiving either the quadroma-derived BsAb OKT3 × HD37 or the CD3 × CD19 diabody, both with or without the anti-CD28 mAb 15E8 (calculated *p1* values were 0.445 and 0.5301, respectively; *p2* values were 0.5734 and 0.6164, respectively; Fig. 6). The comparison with the control groups demonstrated no significant delay in the starting point of tumor growth in mice receiving both the BsAb and diabody ($p1 = 0.2125$), but the tumors grew significantly slower in those groups ($p2 < 0.0001$). In contrast, animals receiving the BsAb or diabody in combination with anti-CD28 mAb revealed a significant delay in the starting point of tumor growth and slower tumor growth rates as compared with control groups ($p1 < 0.0001$, $p2 < 0.0001$). Finally, the use of the costimulatory mAb 15E8 led to a delay in the starting point of tumor growth in comparison with that of animals receiving BsAb or diabody without CD28 costimulation ($p1 < 0.0001$), but had no effect on the tumor growth rate ($p2 = 0.1301$). The animals were sacrificed when the tumors achieved diameters of 15 mm. Sacrifice dates were recorded, and the mean survival in each group was calculated. Mean survival was 37.2 ± 1.5 days in the control group receiving PBS, 38.2 ± 1.9 in the control group receiving human PBL alone, 71.5 ± 1.5 days in the group given BsAb OKT3 × HD37, and 75.4 ± 2.2 days in the diabody-treated group. The addition of the anti-CD28 mAb 15E8 resulted in a prolonged survival time for both BsAb- and diabody-treated animals. The calculated mean survival was 87.2 ± 1.6 days in the group given BsAb OKT3 × HD37 plus mAb 15E8 and 89.6 ± 2.2 days for animals receiving bispecific diabody in combination with anti-CD28 mAb. The statistical analysis of survival times demonstrated significant differences between all the therapy groups and the control groups ($p = 0.002$ in each case). In contrast, there was no significant difference in survival between the animals treated either with BsAb OKT3 × HD37 or diabody both with and without CD28 costimulation ($p = 0.0925$ and 0.1421, respectively).

Discussion

The treatment of leukemias and malignant lymphoma includes multiple courses of polychemotherapy and/or radiotherapy, but, despite aggressive treatment, a fairly large number of patients relapse and most remissions cannot be extended beyond minimal residual disease. An emerging alternative approach is the retargeting of cellular effector systems such as T cells, NK cells, or FcγR-positive cells (granulocytes, macrophages) by BsAbs (43–45). However, the progress of these immunotherapeutic agents into clinical applications has been slow, mainly due to the low yields of clinical grade bispecific molecules and the immunogenicity of murine BsAbs.

More recently, recombinant bispecific diabodies have been created that only comprise Ab V domains (16, 17, 19). They are less immunogenic than quadroma-derived BsAb and can be easily produced in bacteria in relatively high yields.

The CD3 × CD19 diabody described in the present investigation was designed for treating of minimal residual disease in patients with B cell malignancies. It bound to target B cells more strongly than to effector T cells, due to the almost 10-fold higher affinity of the anti-CD19 moiety. Such relatively strong binding to a target tumor cell and weaker binding to an effector cell may have certain advantages for tumor therapy. Artificial signaling via the CD3 Ag mimics the physiological Ag-specific activation of T lymphocytes by MHC-bound Ag. Accordingly, in a model of TCR serial triggering (46), a high off-rate of the TCR is essential because it allows a single peptide-MHC complex to engage many TCRs in successive rounds of ligation, triggering, and dissociation. Therefore, a high affinity due to a low off-rate may inhibit TCR reuse.

As expected, the diabody had lower clearance rates compared with the smaller scFv Ab fragment. It proved to be fairly stable both in vitro and in vivo, although it is formed by the noncovalent association of two hybrid scFv fragments. A comparison of the diabody $t_{1/2}$ under different experimental conditions (Table II) indicated that the therapeutic dose will be mainly determined by its clearance from the bloodstream, and not by degradation.

In vitro experiments demonstrated that the CD3 × CD19 diabody was able to activate T cells in the presence of irradiated tumor cells. It was also able to effectively recruit T cells for killing both $CD19^+$ Raji cells and fresh CLL leukemia cells from a patient. The cytolytic potential of the diabody was enhanced in the presence of anti-CD28 mAb, which induces a well-known costimulatory signal (for review, see Ref. 47). T cell signaling via the TCR alone without a costimulatory signal can lead to anergy (48) or even activation-induced cell death (49). Furthermore, recent experiments demonstrated that costimulation via CD28 together with anti-CD3 × antitumor BsAb prevents apoptosis in the targeted T cells (50) and even reactivates exhausted tumor-reactive CTL in an in vivo model of human melanoma (42). Impressive results were also obtained using anti-CD28 × antitumor BsAb together with anti-CD3 × antitumor BsAb for curing human primary tumors (43) and metastases (42) in immunodeficient mice. However, it is

somewhat surprising that the anti-CD28 mAb improves the efficacy of B cell lysis in our system because the Raji B cells carry both costimulatory molecules CD80 and CD86. In vitro, the anti-CD28 mAb in solution probably saturates the CD28 molecules on the effector cells faster and more efficiently than the cell-bound B7 of the target cells. A similar situation may occur in our in vivo model, in which the circulating anti-CD28 mAb can bind to the CD28 molecule more effectively than the B7 present only at the tumor site.

The ultimate goal of any antitumor immunotherapy is the in vivo eradication of tumor cells. Recently, we described a CD30 × CD16 diabody that was able to induce a marked regression of xenotransplanted human Hodgkin's lymphoma in SCID mice due to the recruitment of human NK cells (51). In the present study, we tested the potency of a CD3 × CD19 diabody to mediate T cell-dependent tumor lysis in a fairly stringent in vivo model of immunodeficient mice bearing a s.c. growing human B cell lymphoma. The number of Raji cells used for s.c. tumor growth was much higher than in other studies (50), and we let the malignant cells grow for 5 days before starting treatment. A prolonged T cell-dependent suppression of tumor growth was induced by the diabody. Mice receiving the diabody had a longer mean survival time twice as long as the control animals. The administration of the diabody together with the anti-CD28 mAb further prolonged survival. The comparable efficiency of the diabody compared with the quadroma-derived BsAb was very surprising in view of its relatively rapid clearance through the kidneys. This is probably compensated by a better tumor penetration and more efficient induction of cell lysis.

The successful use of a recombinant anti-Id × anti-mouse CD3 scFv-scFv tandem ($(scFv)_2$) for the in vivo treatment of a murine B cell lymphoma has recently been described (52). However, the yield of correctly folded $(scFv)_2$ molecules is generally quite low, at least in the periplasm of bacteria (19). Therefore, for our work, we chose to make a bispecific diabody, which is formed by the noncovalent association of two scFvs and has a stable compact structure (18). To our knowledge, these results describe the first in vivo application of bispecific diabodies to redirect human cytotoxic T cells. The efficacy of the diabody's immune recruiting capacity suggests that it can be used to replace the bispecific mAb for immunotherapy. This would facilitate larger clinical trials with more extensive cycles of treatments, because it is less immunogenic and can probably be produced and purified at relatively low cost.

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References

1. Fanger, M. W., P. M. Morganelli, and P. M. Guyre. 1992. Bispecific antibodies. *Crit. Rev. Immunol. 12:101.*
2. Canevari, S., G. Stoter, F. Arienti, G. Bolis, M. I. Colnaghi, E. M. D. Re, A. M. M. Eggermont, S. H. Goey, J. W. Grazama, C. H. J. Lamers, et al. 1995. Regression of advanced ovarian carcinoma by intraperitoneal treatment with autologous T lymphocytes retargeted by a bispecific monoclonal antibody. *J. Natl. Cancer Inst. 87:1463.*
3. Nitta, T., K. Sato, H. Yagita, K. Okumura, and S. Ishi. 1990. Preliminary trial of specific targeting therapy against malignant glioma. *Lancet 335:368.*
4. Grossbard, M. L., O. W. Press, F. R. Appelbaum, I. D. Bernstein, and L. M. Nadler. 1992. Monoclonal antibody-based therapies of leukemia and lymphoma. *Blood 80:863.*
5. Bohlen, H., T. Hopff, O. Manzke, A. Engert, D. Kube, P. D. Wickramanayake, V. Diehl, and H. Tesch. 1993. Lysis of malignant B cells from patients with B-chronic lymphocytic leukemia by autologous T cells activated with CD3 × CD19 bispecific antibodies in combination with bivalent CD28 antibodies. *Blood 82:1803.*
6. Brennan, M., P. F. Davidson, and H. Paulus. 1985. Preparation of bispecific antibodies by chemical recombination of monoclonal immunoglobulin G1 fragments. *Science 229:81.*
7. Glennie, M. J., H. M. McBride, A. T. Worth, and G. T. Stevenson. 1987. Preparation and performance of bispecific $F(ab'\gamma)_2$ antibody containing thioether-linked $Fab'\gamma$ fragments. *J. Immunol. 139:2367.*
8. Khazaeli, M. B., R. M. Conry, and A. F. LoBuglio. 1994. Human immune response to monoclonal antibodies. *J. Immunother. 15:42.*
9. Carter, P., J. Ridgway, and Z. Zhu. 1995. Toward the production of bispecific antibody fragments for clinical applications. *J. Hematother. 4:463.*
10. Plückthun, A., and P. Pack. 1997. New protein engineering approaches to multivalent and bispecific antibody fragments. *Immunotechnology 3:83.*
11. De Kruif, J., and T. Logtenberg. 1996. Leucine zipper dimerized bivalent and bispecific scFv antibodies from a semi-synthetic antibody phage display library. *J. Biol. Chem. 271:7630.*
12. Müller, K. M., K. M. Arndt, W. Strittmatter, and A. Plückthun. 1998. The first constant domain (C_H1 and C_L) of an antibody used as heterodimerization domain for bispecific miniantibodies. *FEBS Lett. 422:259.*
13. Gruber, M., B. A. Schodin, E. R. Wilson, and D. M. Kranz. 1994. Efficient tumor cell lysis mediated by a bispecific single chain antibody expressed in *Escherichia coli. J. Immunol. 152:5368.*
14. Kurucz, I., J. A. Titus, C. R. Jost, C. M. Jacobus, and D. M. Segal. 1995. Retargeting of CTL by an efficiently refolded bispecific single-chain Fv dimer produced in bacteria. *J. Immunol. 154:4576.*
15. Holliger, P., T. Prospero, and G. Winter. 1993. "Diabodies": small bivalent and bispecific antibody fragments. *Proc. Natl. Acad. Sci. USA 90:6444.*
16. Holliger, P., J. Brissinck, R. L. Williams, K. Thielemans, and G. Winter. 1996. Specific killing of lymphoma cells by cytotoxic T-cells mediated by a bispecific diabody. *Protein Eng. 9:299.*
17. Zhu, Z., G. Zapata, R. Shalaby, B. Snedecor, H. Chen, and P. Carter. 1996. High level secretion of a humanized bispecific diabody from *Escherichia coli. Bio/Technology 14:192.*
18. Perisic, O., P. A. Webb, P. Holliger, G. Winter, and R. L. Williams. 1994. Crystal structure of a diabody, a bivalent antibody fragment. *Structure 2:1217.*
19. Kipriyanov, S. M., G. Moldenhauer, G. Strauss, and M. Little. 1998. Bispecific CD3 × CD19 diabody for T cell-mediated lysis of malignant human B cells. *Int. J. Cancer 77:763.*
20. Horn, U., W. Strittmatter, A. Krebber, U. Knupfer, M. Kujau, R. Wenderoth, K. Müller, S. Matzku, A. Plückthun, and D. Riesenberg. 1996. High volumetric yields of functional dimeric miniantibodies in *Escherichia coli*, using an optimized expression vector and high-cell-density fermentation under non-limited growth conditions. *Appl. Microbiol. Biotechnol. 46:524.*
21. Bothmann, H., and A. Plückthun. 1998. Selection for a periplasmic factor improving phage display and functional periplasmic expression. *Nat. Biotechnol. 16:376.*
22. Holck, A., and K. Kleppe. 1988. Cloning and sequencing of the gene for the DNA-binding 17K protein of *Escherichia coli. Gene 67:117.*
23. Thisted, T., N. S. Sorensen, E. G. Wagner, and K. Gerdes. 1994. Mechanism of post-segregational killing: Sok antisense RNA interacts with Hok mRNA via its 5′-end single-stranded leader and competes with the 3′-end of Hok mRNA for binding to the *mok* translational initiation region. *EMBO J. 13:1960.*
24. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA 74:5463.*
25. Maurer, R., B. Meyer, and M. Ptashne. 1980. Gene regulation at the right operator (O_R) bacteriophage λ. I. O_R3 and autogenous negative control by repressor. *J. Mol. Biol. 139:147.*
26. Blackwell, J. R., and R. Horgan. 1991. A novel strategy for production of a highly expressed recombinant protein in an active form. *FEBS Lett. 295:10.*
27. Kipriyanov, S. M., G. Moldenhauer, and M. Little. 1997. High level production of soluble single chain antibodies in small-scale *Escherichia coli* cultures. *J. Immunol. Methods 200:69.*
28. Kipriyanov, S. M., G. Moldenhauer, A. C. R. Martin, O. A. Kupriyanova, and M. Little. 1997. Two amino acid mutations in an anti-human CD3 single chain Fv antibody fragment that affect the yield on bacterial secretion but not the affinity. *Protein Eng. 10:445.*
29. Kipriyanov, S. M., O. A. Kupriyanova, M. Little, and G. Moldenhauer. 1996. Rapid detection of recombinant antibody fragments directed against cell-surface antigens by flow cytometry. *J. Immunol. Methods 196:51.*
30. Schodin, B. A., and D. M. Kranz. 1993. Binding affinity and inhibitory properties of a single-chain anti-T cell receptor antibody. *J. Biol. Chem. 268:25722.*
31. Adair, J. R., D. S. Athwal, M. Bodmer, S. M. Bright, A. Collins, V. L. Pulito, P. E. Rao, R. Reedman, A. L. Rothermel, D. Xu, et al. 1994. Humanization of the murine anti-human CD3 monoclonal antibody OKT3. *Hum. Antibodies Hybridomas 5:41.*
32. Adams, G. P., R. Schier, K. Marshall, E. J. Wolf, A. M. McCall, J. D. Marks, and L. M. Weiner. 1998. Increased affinity leads to improved selective tumor delivery of single-chain Fv antibodies. *Cancer Res. 58:485.*
33. Matzinger, P. 1991. The JAM test: a simple assay for DNA fragmentation and cell death. *J. Immunol. Methods 145:185.*
34. Kipriyanov, S. M., S. Dübel, F. Breitling, R. E. Kontermann, and M. Little. 1994. Recombinant single-chain Fv fragments carrying C-terminal cysteine residues: production of bivalent and biotinylated miniantibodies. *Mol. Immunol. 31:1047.*
35. Csóka, M., G. Strauss, K.-M. Debatin, and G. Moldenhauer. 1996. Activation of T cell cytotoxicity against autologous common acute lymphoblastic leukemia (cALL) blasts by CD3 × CD19 bispecific antibody. *Leukemia 10:1765.*

36. Davidian, M., and D. Giltinan. 1995. *Nonlinear Models for Repeated Measurement Data*. Chapman and Hall.
37. Pinheiro, J. C., and D. M. Bates. 2000. Mixed-effects models. In *Statistics and Computing Series*. Springer-Verlag, New York.
38. Pezzutto, A., B. Dörken, P. S. Rabinovitch, J. A. Ledbetter, G. Moldenhauer, and E. A. Clark. 1987. CD19 monoclonal antibody HD37 inhibits anti-immunoglobulin-induced B cell activation and proliferation. *J. Immunol. 138:2793.*
39. Kung, P. C., G. Golstein, E. L. Reinherz, and S. F. Schlossman. 1979. Monoclonal antibodies defining distinctive human T cell surface antigens. *Science 206:347.*
40. Le Gall, F., S. M. Kipriyanov, G. Moldenhauer, and M. Little. 1999. Di-, tri- and tetrameric single chain Fv antibody fragments against human CD19: effect of valency on cell binding. *FEBS Lett. 453:164.*
41. Hombach, A., T. Tillmann, M. Jensen, C. Heuser, R. Sircar, V. Diehl, W. Kruis, and C. Pohl. 1997. Specific activation of resting T cells against CA19–9^+ tumor cells by an anti-CD3/CA19-9 bispecific antibody in combination with a costimulatory anti-CD28 antibody. *J. Immunother. 20:325.*
42. Cochlovius, B., A. Perschl, G. J. Adema, and M. Zoller. 1999. Human melanoma therapy in the SCID mouse: in vivo targeting and reactivation of melanoma-specific cytotoxic T cells by bi-specific antibody fragments. *Int. J. Cancer 81:486.*
43. Renner, C., W. Jung, U. Sahin, R. Denfeld, C. Pohl, L. Trumper, F. Hartmann, V. Diehl, R. van Lier, and M. Pfreundschuh. 1994. Cure of xenografted human tumors by bispecific monoclonal antibodies and human T cells. *Science 264:833.*
44. Hartmann, F., C. Renner, W. Jung, C. Deisting, M. Juwana, B. Eichentopf, M. Kloft, and M. Pfreundschuh. 1997. Treatment of refractory Hodgkin's disease with an anti-CD16/CD30 bispecific antibody. *Blood 89:2042.*
45. Stockmeyer, B., T. Valerius, R. Repp, I. A. Heijnen, H. J. Buhring, Y. M. Deo, J. R. Kalden, M. Gramatzki, and J. G. van de Winkel. 1997. Preclinical studies with FcγR bispecific antibodies and granulocyte colony-stimulating factor-primed neutrophils as effector cells against HER-2/*neu* overexpressing breast cancer. *Cancer Res. 57:696.*
46. Valitutti, S., S. Muller, M. Cella, E. Padovan, and A. Lanzavecchia. 1995. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature 375:148.*
47. Chambers, C. A., and J. P. Allison. 1999. Costimulatory regulation of T cell function. *Curr. Opin. Cell Biol. 11:203.*
48. Schwartz, R. H. 1990. A cell culture model for T lymphocyte clonal anergy. *Science 248:1349.*
49. Daniel, P. T., A. Kroidl, S. Cayeux, R. Bargou, T. Blankenstein, and B. Dorken. 1997. Costimulatory signals through B7.1/CD28 prevent T cell apoptosis during target cell lysis. *J. Immunol. 159:3808.*
50. Daniel, P. T., A. Kroidl, J. Kopp, I. Sturm, G. Moldenhauer, B. Dorken, and A. Pezzutto. 1998. Immunotherapy of B-cell lymphoma with CD3 × 19 bispecific antibodies: costimulation via CD28 prevents "veto" apoptosis of antibody-targeted cytotoxic T cells. *Blood 92:4750.*
51. Arndt, M. A., J. Krauss, S. M. Kipriyanov, M. Pfreundschuh, and M. Little. 1999. A bispecific diabody that mediates natural killer cell cytotoxicity against xenotransplanted human Hodgkin's tumors. *Blood 94:2562.*
52. De Jonge, J., C. Heirman, M. de Veerman, S. Van Meirvenne, M. Moser, O. Leo, and K. Thielemans. 1998. In vivo retargeting of T cell effector function by recombinant bispecific single chain Fv (anti-CD3 × anti-idiotype) induces long-term survival in the murine BCL1 lymphoma model. *J. Immunol. 161:1454.*