Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 7021–7025, July 1995
Immunology

A small bispecific antibody construct expressed as a functional single-chain molecule with high tumor cell cytotoxicity

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Communicated by Gunter Blobel, The Rockefeller University, New York, NY, April 14, 1995

ABSTRACT **Construction of a bispecific single-chain antibody derivative is described that consists of two different single-chain Fv fragments joined through a Gly-Ser linker. One specificity of the two Fv fragments is directed against the CD3 antigen of human T cells and the other is directed against the epithelial 17-1A antigen; the latter had been found in a clinical trial to be a suitable target for antibody therapy of minimal residual colorectal cancer. The construct could be expressed in CHO cells as a fully functional protein, while its periplasmic expression in *Escherichia coli* resulted in a nonfunctional protein only. The antigen-binding properties of the bispecific single-chain antibody are indistinguishable from those of the corresponding univalent single-chain Fv fragments. By redirecting human peripheral T lymphocytes against 17-1A-positive tumor cells, the bispecific antibody proved to be highly cytotoxic at nanomolar concentrations as demonstrated by ^{51}Cr release assay on various cell lines. The described bispecific construct has a molecular mass of 60 kDa and can be easily purified by its C-terminal histidine tail on a Ni-NTA chromatography column. As bispecific antibodies have already been shown to be effective *in vivo* in experimental tumor systems as well as in phase-one clinical trials, the small CD3/17-1A-bispecific antibody may be more efficacious than intact antibodies against minimal residual cancer cells.**

The 17-1A or EpCAM antigen, a surface glycoprotein expressed by cells of simple epithelia, has been shown to be a rewarding target for monoclonal antibody therapy against disseminated tumor cells in patients with minimal residual colorectal cancer (1). Since treatment with intact anti-17-1A antibody decreased the 5-year mortality rate of the treated patients by only 30%, we tried to improve the efficacy of this approach by designing a bispecific antibody, which could engage cytotoxic T cells against isolated metastatic 17-1A-positive tumor cells. Target cell-bound bispecific antibodies are known to trigger cytotoxic activity of T lymphocytes by crosslinking of CD3 (2, 3), irrespective of T-cell receptor specificity, major histocompatibility complex (MHC) restriction, or MHC down regulation on tumor cells (4). Additional activation mediated by costimulatory molecules such as CD28 and CD2 has been shown to increase target cell lysis (5, 6). Both *in vitro* and *in vivo* results indicate that bispecific antibodies in general are more effective in tumor cell elimination than antibody-dependent cellular cytotoxicity or complement activity of monoclonal antibodies (7, 8), the latter of which is inhibited by membrane-bound proteins such as CD59 or decay accelerating factor, controlling the cytolytic effect of complement. So far, approaches to construct bispecific antibodies like hybrid hybridomas (9, 10), chemical linkage, renaturation from bacterial inclusion bodies (11, 12), or the use of noncovalent coupling in diabodies (13) or Jun-Fos constructs (14) suffer from low yields, occurrence of ill-defined by-products, or laborious purification procedures. To overcome these problems, we developed a procedure by which two single-chain Fv (sc-Fv) fragments (15, 16) directed at the 17-1A antigen and the CD3 antigen on T lymphocytes were linked by one or three Gly_4-Ser_1 units. The construct could be expressed in CHO cells as one functional single-chain molecule. An N-terminal Flag epitope was inserted for easy detection and a C-terminal histidine tail was attached for efficient purification from culture supernatants. The resulting recombinant protein proved to be highly cytotoxic for tumor cells at nanomolar concentrations. It thus appears as the ideal candidate for therapy of disseminated 17-1A-positive tumor cells during early phases of metastasis when these cells are lodging in interstitial tissue compartments easily accessible for macromolecules as well as for the required effector T cells.

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MATERIALS AND METHODS

Cloning of Variable (V) Immunoglobulin Domains. The V light-chain (V_L) and V heavy-chain (V_H) domains from the M79 hybridoma (anti-17-1A) (17) were cloned according to the standard PCR methods as described by Orlandi *et al.* (18). cDNA synthesis was carried out with random hexamers (Boehringer Mannheim) and SuperScript reverse transcriptase (GIBCO). For amplification of the V domains via PCR with Pfu polymerase, we used the two primers 5′lightEco5 and 3′lightBgl2, flanking the light chain, and 5′heavyEco5 and 3′heavyBspE1, flanking the heavy chain. For detailed specification of primers, see below. Two independent clones of each V domain were sequenced and compared for identity.

The cDNA of the anti-CD3 sc-Fv fragment was kindly provided by A. Traunecker (19).

Construction of Univalent Single-Chain Fragments and Their Periplasmic Expression in *Escherichia coli*. V_L and V_H cDNA isolated from the M79 hybridoma were joined to a single-chain fragment using the standard $(Gly_4\text{-}Ser_1)_3$ linker. For this purpose, a two-step fusion PCR (Pfu polymerase) was performed. The first PCR step introduced a 3′-terminal $(Gly_4\text{-}Ser_1)_2$ coding sequence into V_L with the two primers 5′lightEco5 and 3′lightLinker and a 5′-terminal $(Gly_4\text{-}Ser_1)_2$ coding sequence into V_H with the primers 5′heavyLinker and 3′heavyBspE1. The purified amplification products of V_L and V_H were used for the second step fusion PCR (eight cycles) with the primers 5′lightEco5 and 3′heavyBspE1. The resulting PCR fragment of the single-chain molecule was subcloned (*Eco*RV and *Bsp*EI) into a bacterial expression vector and sequenced. A PCR fragment of the anti-CD3 sc-Fv fragment obtained with the primers 5′heavyCD3linker5 and 3′lightCD3His (*Taq* polymerase) was also subcloned into the bacterial expression vector with *Eco*RV and *Hin*dIII and sequenced. The vector for periplasmic expression in bacteria, provided by A. Plückthun (Zürich), consists of an isopropyl β-D-thiogalactopyranoside-inducible *lac* promoter, the

Abbreviations: sc-Fv fragment, single-chain Fv fragment; bsc-Ab, bispecific single-chain antibody with Gly_4Ser_1 linker; V domain, variable domain; V_H and V_L, V domain heavy and light chains; DHFR, dihydrofolate reductase; PBMC, peripheral blood mononuclear cell; FACS, fluorescence-activated cell sorter.

periplasmic signal sequence OmpA,and a 5′-terminal Flag epitope (Kodak) for detection. The periplasmic expression was performed in *E. coli* strain JM83 according to the procedure described by Plückthun *et al.* (20).

Construction of Bispecific Single-Chain Fragments and Eukaryotic Expression. Construction of the bispecific single-chain antibody was performed in three steps (Fig. 1).

(*i*) To introduce the Gly_4-Ser_1 or $(Gly_4\text{-}Ser_1)_3$ linker sequences between the V_H regions of the M79 sc-Fv fragment and the anti-CD3 sc-Fv fragment and in order to add a 3′-terminal histidine tail, we generated a PCR fragment of the anti-CD3 sc-Fv fragment DNA with the two primers 5′heavyCD3linker5 and 5′heavyCD3linker15 and with 3′lightCD3His (*Taq* polymerase) and subcloned it with the *Bsp*EI and *Hin*dIII restriction enzymes into the vector already containing the M79 sc-Fv fragment.

(*ii*) A synthetic DNA oligodimer (5′leaderFlag and 3′leaderFlag) coding for a eukaryotic secretory signal sequence together with the Flag epitope was subcloned into the same vector with the *Xba* I and *Eco*RV enzymes. We anticipated that a protein beginning with the Flag epitope would be secreted by eukaryotic cells (21).

(*iii*) Finally, the bsc-Ab fragment was subcloned into a vector for stable eukaryotic expression. This vector contained the promoter of human elongation factor 1α, kindly provided by S. Nagata (22), followed by a multiple cloning site and an internal ribosomal binding site (23), which allows bicistronic expression of the construct and dihydrofolate reductase (DHFR) used as a selection marker (P.K., unpublished data). The expression was performed in DHFR-deficient CHO cells as described by Kaufman (24). The cells were transfected by electroporation and grown for selection in nucleoside-free α-MEM supplemented with dialyzed 10% fetal calf serum (FCS) (GIBCO) and 2 mM L-glutamine. To increase the expression rate by gene amplification, the transfectants were subsequently exposed to 20 nM methotrexate. For production of bsc-Ab cells were grown in roller bottles (Falcon).

List of Primers. The following primers were used: 5′light-EcoV, 5′-aagatatccagctgacccagtctcca-3′; 3′lightBgl2, 5′-gttagatctcgagcttggtccc-3′; 5′heavyEcoV, 5′-aagatatcaggtsmarctgcagsagtcwgg-3′ (s = c or g, m = a or c, r = a or g, w = a or t); 3′heavyBspE1, 5′-aatccggaggagacggtgaccgtggtcccttggccccag-3′; 3′lightlinker, 5′-ggagccgccgccgccagaaccaccaccaccttgatctcgagcttggtccc-3′; 5′heavylinker, 5′-ggcggcggcggctccggtggtggtggttctcaggtgaaactgcaggagtc-3′; 5′heavyCD3linker5, 5′-taatccggaggtggtggatccgatatcaaactgcagcagtcagg-3′; 5′heavyCD3linker15, 5′-taatccggaggtggtggttccgggggtggtggttccgggggtggtggatccgatatcaaactgcagcagtcagg-3′; 3′lightCD3His, 5′-tttaagcttgtcgactaatgatgatggtgatgatgtttcagctccagcttggtcccagc-3′; 5′leaderFlag, 5′-ctagaaattccaccatgggatggagctgtatcatcctcttcttggtagcaacagctacggtgtccactccgactacaaagatgatgacgataaggat-3′; 3′leaderFlag, 5′-atccttatcgtcatcatctttgtagtcggagtggacacctgtagctgttgctaccaagaagaggatgatacagctccatcccatggtggaatt-3′.

Purification with Flag Tag and Histidine Tail. For purification with the Flag M1 affinity column (Kodak) we dialyzed the periplasmic fraction of the bacterial lysates against optimal buffer (0.15 M NaCl/0.01 M sodium phosphate/1.0 mM $CaCl_2$, pH 7.4). To the supernatant from CHO transfectants we added $CaCl_2$ to a final concentration of 1.0 mM. For further steps, we followed the suggested protocol (Kodak). The bsc-Ab containing a histidine tail was purified by affinity chromatography on a Ni-NTA column (Quiagen). The bound bsc-Ab was eluted with a gradient of imidazole from 0 to 500 mM increasing in 100 mM steps. Elution occurred at a concentration of 200 mM imidazole as a distinct peak. The eluted proteins were dialyzed against phosphate-buffered saline (PBS) and sterile-filtrated.

Cytofluorometry, ELISA, Western Blot. For cytofluorometric analysis (Becton Dickinson), 10^6 cells were incubated in 50 μl of medium with 50 μl of antibody solutions containing the relevant constructs at different concentrations. Bound antibody constructs were detected with Flag M1 antibody (Kodak) and a fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (F313; Dako). Cells incubated with PBS and stained with Flag M1 and F313 antibodies served as a negative control. Specific fluorescence was calculated as (mean channel fluorescence, experimental) − (mean channel fluorescence, negative control).

For detection with an ELISA, recombinant soluble 17-1A antigen purified from CHO cells transfected with cDNA coding for the extracellular part of 17-1A (ref. 25; P.K., unpublished data) (10 μg/ml) was coated to ELISA plates. Bound antibody constructs were detected with Flag M2 antibody (Kodak) amplified by peroxidase-conjugated rabbit anti-mouse IgG (P260; Dako). The colored precipitate was measured in an ELISA reader. For negative controls, the plates were incubated with PBS instead of constructs. Specific OD was calculated as (OD, experimental) − (OD, negative control).

Western blotting was performed with Flag M1 and the peroxidase-labeled antibody P260.

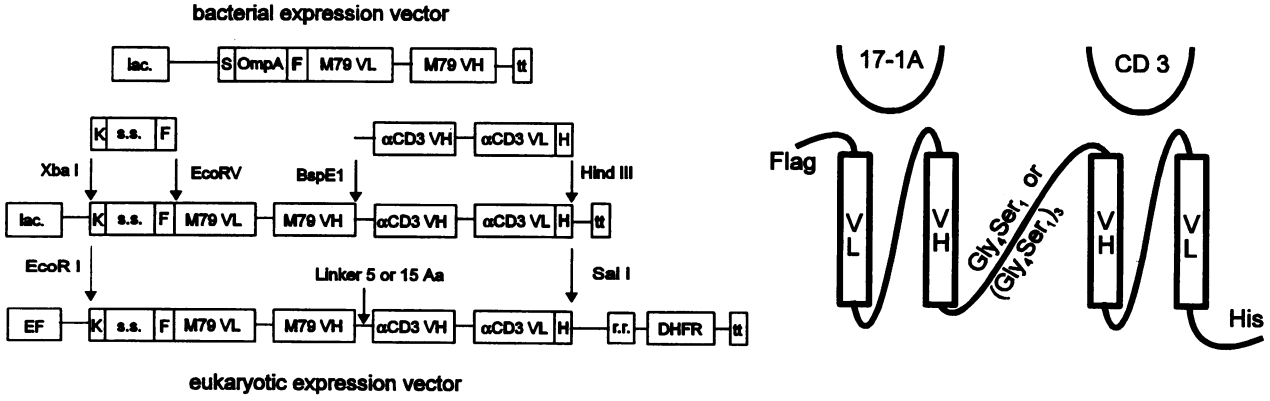

FIG. 1. (*Left*) Design and various cloning steps leading to the final bispecific construct. The bacterial expression vector for the M79 single-chain fragment (above) contains a lac promoter (lac.), a Shine–Dalgarno sequence (S), a periplasmic signal sequence (OmpA), and a Flag sequence (F). The bacterial S and OmpA segments were substituted by a eukaryotic secretory signal sequence (s.s.) including a 5′-terminal Kozak site (K) and a 3′-terminal Flag sequence (F). Into this vector the anti-CD3 (αCD3) sc-Fv fragment cDNA was subcloned, which carried a 5′-terminal Gly_4-Ser_1 or $(Gly_4\text{-}Ser_1)_3$ linker sequence and a 3′-terminal tail of 6 histidine residues (H). The resulting bsc-Ab DNA was subcloned into *Eco*RI and *Sal* I (partial) into the described eukaryotic expression vector. EF, promoter of human elongation factor 1α; rr, ribosomal reinitiation site; tt, transcription termination or poly(A) signal; Aa, amino acids. (*Right*) Scheme of the bsc-Ab as it is secreted by CHO cells into the supernatant.

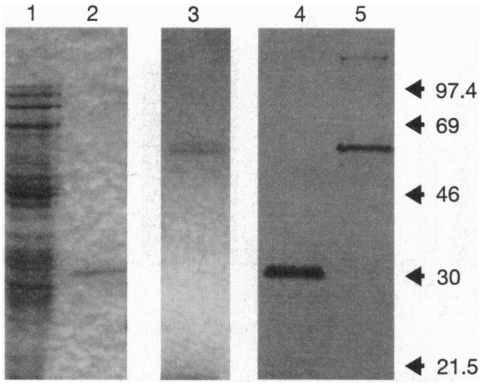

FIG. 2. SDS/PAGE and Western blotting. Lane 1, Coomassie stain of the crude bacterial periplasm lysate, from which the M79 sc-Fv fragment was purified (lane 2). Lane 3, Coomassie stain of the purified bsc-Ab. A Western blot of the purified M79 sc-Fv fragment and bsc-Ab is shown in lanes 4 and 5. Molecular mass (kDa) is indicated on the right.

Cell Lines. X63 cells were transfected with 17-1A cDNA (25). All other cell lines were obtained from ATCC. Kato and HT-29 are 17-1A-positive cell lines. Jurkat is a CD3-positive T-cell line, while X63, K562, and U937 are negative for both surface molecules.

Cytotoxicity Assay. For ^{51}Cr release assay human peripheral blood mononuclear cells (PBMCs) as effector cells were isolated from the fresh buffy coat of random donors. The PBMCs were separated by Ficoll density-gradient centrifugation (Pharmacia) with a subsequent $100 \times g$ centrifugation step to remove thrombocytes. Unstimulated PBMCs (5×10^5 cells) were added in a volume of 100 μl of RPMI 1640 medium (Sigma) with 10% FCS (GIBCO) to each well of a flat-bottomed microtiter plate (Costar) and incubated overnight at 37°C. Target cells were labeled for 2 h with ^{51}Cr. Labeled target cells (100 μl) and bsc-Ab in different concentrations (50 μl) were added to the PBMCs and incubated for 4 or 20 h at 37°C. PBS instead of bsc-Ab was used as a negative control. Maximal release was determined by lysis of target cells with Maly buffer (2% SDS/0.37% EDTA/0.53% Na_2CO_3). Spontanous ^{51}Cr release was determined for target cells incubated without effector cells or antibody. Incubation of target cells with bsc-Ab at the highest concentration did not result in measurable lysis. Specific lysis was calculated as [(cpm, experimental release) − (cpm, spontaneous release)]/[(cpm, maximal release) − (cpm, spontaneous release)]. Triplicate samples were measured for each antibody concentration and effector/target cell ratio, and every experiment has been reproduced several times.

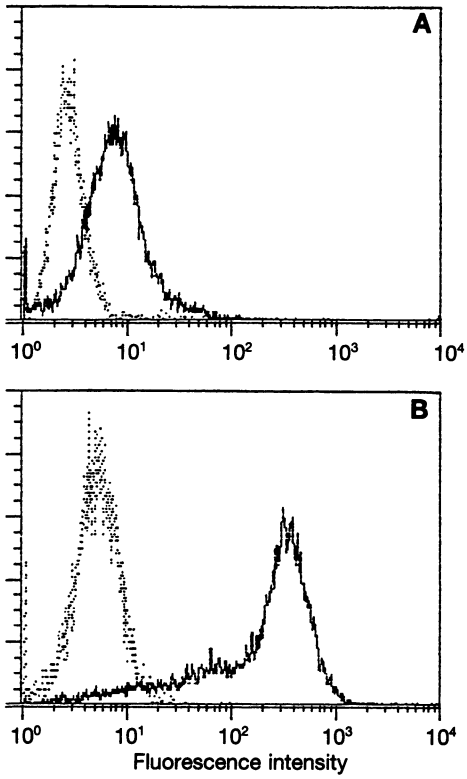

FIG. 3. FACS analysis with the bsc-Ab (250 μg/ml) on CD3-positive Jurkat cells (*A*) and on 17-1A-positive Kato cells (*B*). Broken lines are negative controls.

RESULTS

SDS/PAGE and Western Blot. Purification of bsc-Ab from the supernatant of transfected CHO cells (20 nM methotrexate) yielded 12–15 mg/liter. The bsc-Ab was eluted from the Ni-NTA column as a distinct peak at a concentration of 200 mM imidazole. The univalent anti-17-1A sc-Fv fragment and anti-CD3 sc-Fv fragment expressed in *E. coli* were purified using the Flag M1 affinity column with a yield of ≈0.2 mg/200 ml of bacterial culture. The results of SDS/PAGE and Western blot analysis (Fig. 2) show the expected double size of the bsc-Ab (60 kDa) compared to the parent sc-Fv fragments (30 kDa).

Binding Properties. Binding specificities of the bsc-Ab to CD3 and 17-1A were shown by fluorescence-activated cell sorter (FACS) analysis on CD3-positive Jurkat and 17-1A-positive Kato cells (Fig. 3). No binding was detectable on cell lines X63, U937, and K562, all of which are known to express neither 17-1A nor CD3, whereas 17-1A-transfected X63 and 17-1A-positive HT29 cells also bound the bsc-Ab.

The bsc-Ab and the corresponding monovalent sc-Fv fragments expressed in *E. coli* are expected to have similar binding affinities. For comparison, specific mean channel fluorescence on 17-1A-positive Kato cells was plotted against molar concentrations of bsc-Ab or the 17-1A-specific sc-Fv fragment (Fig. 4*A*). The results proved to be almost identical and could be confirmed by ELISA (Fig. 4*B*) with immobilized recombinant soluble 17-1A (P.K., unpublished data). The CD3-binding affinity of the bsc-Ab was found to resemble that of the corresponding anti-CD3 sc-Fv fragment by plotting specific mean channel fluorescence on CD3-positive Jurkat cells against the corresponding concentration values (Fig. 4*C*). The bsc-Ab and the monovalent single-chain fragments were detected in the same way.

Although periplasmic expression in *E. coli* is known to yield functional sc-Fv fragments (20), consisting of two immunoglobulin V domains, addition of a third or fourth V domain by using peptide linkers completely abolished binding activity of periplasmic preparations, despite the presence of recombinant protein in sufficient amounts (data not shown). This shows that the periplasm of *E. coli* is insufficient for functional expression of these antibody derivatives containing more than two immunoglobulin domains on a single polypeptide chain.

Cytotoxic Activity Against 17-1A-Positive Tumor Cells. The anti-17-1A-anti-CD3 bsc-Ab proved to be highly cytotoxic for several tumor cell lines in a ^{51}Cr release assay (Fig. 5). To approximate the *in vivo* conditions, we used unstimulated PBMCs from healthy donors as effector cells (26). Lysis was found to be specific for the target antigen, as 17-1A-negative murine X63 plasmacytoma cells become susceptible to lysis after transfection with 17-1A cDNA. Different cell lines were not equally susceptible to bsc-Ab-mediated lysis, which may be explained by differences in the surface density of the target molecule (i.e., 17-1A-transfected X63 cells), the formation of target cell aggregates (i.e., HT-29), the expression of accessory

molecules (27), or the susceptibility for the cytotoxic mechanisms of T lymphocytes (28).

The kinetics of bsc-mediated cytotoxicity against Kato cells is shown in Fig. 6. According to ref. 26, we assume that only a subpopulation of the peripheral blood lymphocytes can be activated via CD3, so that one effector cell has to lyse several tumor cells with the need of additional time for reattachment and lysis. Further time may be necessary to bring unstimulated T cells into an activated state.

So far, all experiments have been performed with the short linker version of the bsc-Ab. To investigate the influence of the linker length between effector and target specificity on tumor cell lysis, we performed a ^{51}Cr release assay using the long-linker version with an inter-Fv linker consisting of three instead of one Gly_4-Ser_1 units. A longer linker should increase the flexibility of the molecule with a possible influence on specific lysis; however, no difference in tumor cell lysis was detectable in our assays (data not shown).

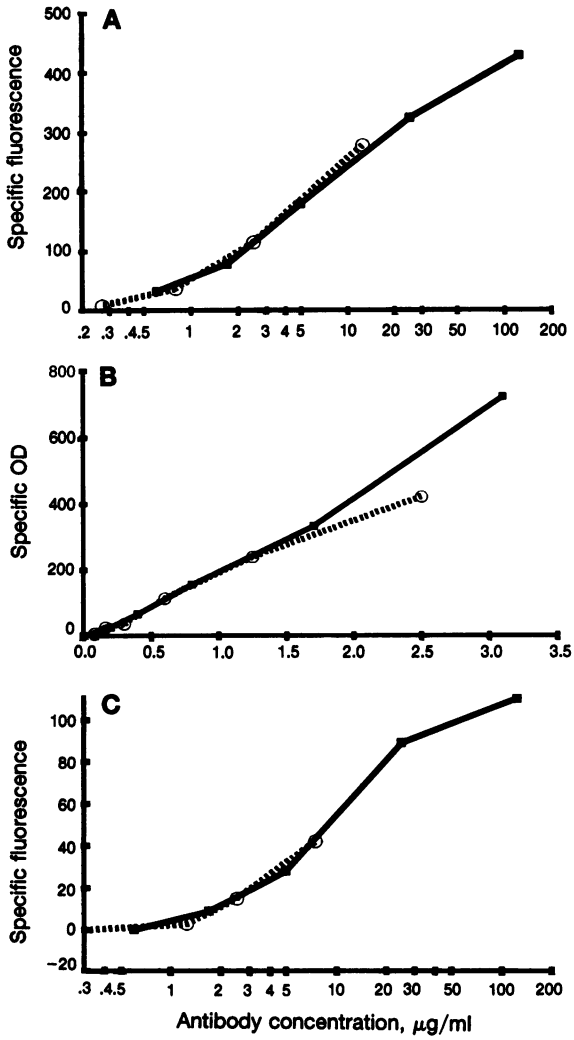

FIG. 4. Comparison of the specific fluorescence signal obtained with the monovalent sc-Fv fragment and the bsc-Ab in different concentrations. (*A*) FACS analysis of M79 sc-Fv fragment and the bsc-Ab on 17-1A-positive Kato cells. (*B*) M79 sc-Fv fragment and bsc-Ab in an ELISA with soluble 17-1A. (*C*) FACS analysis of anti-CD3 sc-Fv fragment and bsc-Ab on Jurkat cells. All sc-Fv fragments are shown as open circles and bsc-Ab are solid squares. Values on the *x* axis refer to concentration of the sc-Fv fragments; for the bsc-Ab these values must be multiplied by 2. This transformation equalizes the molar concentrations of the sc-Fv fragment (30 kDa) and the bsc-Ab (60 kDa) on the *x* axis.

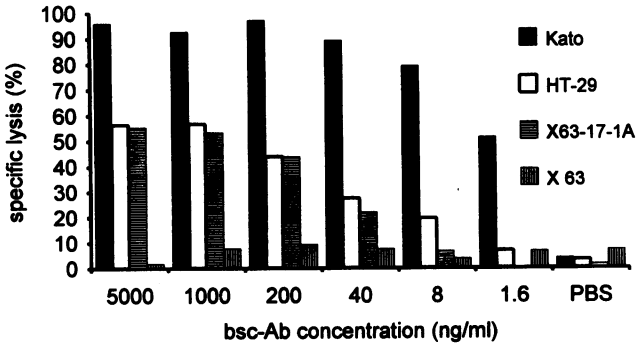

FIG. 5. Cytotoxicity of bsc-Ab in a ^{51}Cr release assay with unstimulated human PBMCs and different cell lines. Effector/target cell ratio, 20:1; incubation time, 20 h.

DISCUSSION

Despite the obvious advantages of speed and cost of protein expression in *E. coli* eukaryotic host cells seem to be preferable for expression of recombinant bsc-Abs in order to overcome the difficulties of conventional techniques of bispecific antibody production. As bsc-Ab molecules are correctly folded during the process of expression and secretion by mammalian cells, the described way of producing bsc-Abs yields a well-defined and homogeneous recombinant protein, which can easily be purified from culture supernatant by using a C-terminal histidine tail.

When compared with bsc-Ab production in *E. coli* (11, 12), the various advantages of the presented eukaryotic expression are conspicuous: (*i*) renaturation of the protein is not necessary, (*ii*) antigen-specific affinity purification required to separate fully active bsc-Ab from inactive or partially active molecules can be avoided (many relevant antigens are not available in sufficient amounts for affinity purification!), and (*iii*) because of the DHFR amplification system, the yield of fully active bsc-Ab (15 mg/liter) exceeds that of bacterial expression.

The one-step procedure described here for generating functional bsc-Abs in CHO cells proved to be generally applicable, as it has been successfully done with other specificities (data not shown). Multispecific antibodies directed to three or more different antigens may now also be produced by the single-chain approach, so that the advantages of eukaryotic expression will become even more evident.

In other experiments, bsc-Ab-mediated cytotoxic activity was apparently not influenced by the length of the linker joining the two V_H-V_L pairs. Therefore, special measures to prevent wrong domain association do not seem to be absolutely necessary. However, in the interest of long-term stability, a short linker between the two different Fv fragments or a linker

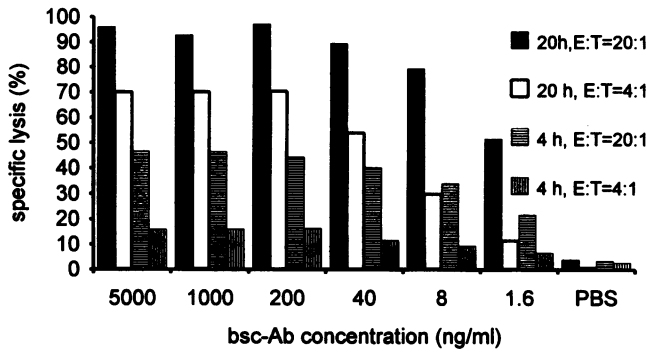

FIG. 6. Cytotoxic activity of bsc-Ab in a ^{51}Cr release assay with Kato cells and unstimulated human PBMCs using different incubation times (20 or 4 h) and different effector/target cell ratios (20:1 or 4:1).

with secondary structure may be preferable. Further stability, as well as avoidance of wrong domain association, can be achieved by introducing intra-Fv disulfide bonds between V_L and V_H domains (29, 30). A domain arrangement designed to sterically separate V_L and V_H domains of different specificities, as the one used here—namely, V_{L1}-V_{H1}-V_{H2}-V_{L2} or alternatively V_{H1}-V_{L1}-V_{L2}-V_{H2}—may be of additional advantage.

The systemic application of bispecific antibodies [$F(ab)_2$ fragments] in cancer patients has been associated with severe side effects (31), which most probably originate from target cell-independent direct activation of T cells and subsequent release of cytokines. *In vitro* studies with peripheral blood lymphocytes have shown that T-cell receptor clustering, a precondition for effective T-cell activation via CD3, is caused by intact anti-CD3 antibodies or immobilized anti-CD3 $F(ab)_2$ fragments (32). The presence of Fc parts in bispecific antibodies is also responsible for lysis of Fc-receptor-positive cells by retargeted T cells (10). A mouse model has shown that unspecific activation of T cells may largely depend on the purity of the bispecific antibodies lacking the Fc part (33). The recombinant production of bispecific single-chain antibodies yields a molecule that consists of V domains only and avoids any part of the antibody molecule that is not necessary for antigen binding. In addition, the low molecular mass of bsc-Ab (60 kDa) facilitates penetration into tumors, as has been shown for Fab or Fv fragments (34).

Experiments with mouse models, using either nude athymic mice with transplanted human tumors (35–37) or immunocompetent mice with syngeneic tumors (7, 8, 38), have demonstrated that T-cell retargeting leads to a significant survival benefit and tumor size reduction. Two clinical trials (39, 40) with autologous lymphocytes precoated with bispecific antibodies have also shown promising results. Therefore, we expect the new anti-17-1A-CD3 bispecific antibody to be an ideal candidate for systemic administration in colorectal cancer patients with disseminated residual tumor cells after complete resection of their primary tumor. In this group of patients, even an intact murine monoclonal antibody led to a significantly increased 5-year survival. Efficient expression of functional bispecific single-chain antibodies in eukaryotic cells should make the bispecific technology more amenable for other applications, which so far have been hampered by the limited availability of these interesting molecules.

We thank A. Traunecker for kindly providing the anti-CD3 single-chain fragment. We are particularly indebted to A. Plückthun and P. Pack for advice and material for the bacterial expression of single chains and E. Kopp for protein purification. This work was supported by grants from Genzentrum München (BMFT), Deutsche Krebshilfe Bonn, and Friedrich Baur-Stiftung, Munich.

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