

CONSTRUCTION AND CHARACTERIZATION OF A BISPECIFIC DIABODY FOR RETARGETING T CELLS TO HUMAN CARCINOMAS

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We describe the construction of a recombinant bispecific antibody fragment in the diabody format with specificity for both the well-established human pancreatic carcinoma associated target antigen EGP2 (epithelial glycoprotein 2, also known as the CO17-1A antigen or KSA) and the CD3 ϵ chain of human TCR/CD3 complex. The murine anti-EGP2 (MOC31) single chain variable fragment (scFv) and the humanized anti-CD3 (Ucht1v9) scFv were cast into a diabody format (designated Dia5v9) using a short 5 amino acid Gly-Ser linker between immunoglobulin heavy-chain and light-chain variable domains. Purification of the poly-histidine tagged Dia5v9 was achieved from extracts of protease deficient *Escherichia coli* by IMAC chromatography. The Dia5v9 diabody showed strong binding to both EGP2 and CD3 in transfected cells. The *in vitro* efficacy of Dia5v9 in mediating tumor cell lysis by interleukin-2 activated human T cells appeared to be similar to that of the hybrid-hybridoma-derived BsF(ab')₂ Bis1 (anti-EGP2/anti-CD3) in a standard 4-hr ⁵¹Cr-release assay. This small and partially humanized recombinant bispecific antibody fragment may be valuable for T-cell-based immunotherapeutic treatment protocols, retargeting activated peripheral blood T lymphocytes to lyse various human carcinomas *in vivo*. *Int. J. Cancer* 76:232–239, 1998.

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Bispecific antibodies (BsAb) have been used for redirecting T cells toward tumor cells in a non-MHC restricted manner by cross-linking cell surface antigens on tumor cells and the CD3-TCR complex on cytotoxic T cells; they also have significant potential for human tumor therapy. Unfortunately, BsAb prepared by hybrid hybridoma technology or via chemical coupling (Glennie *et al.*, 1987) are difficult to prepare in sufficient amounts and purity, which generally hampers their clinical evaluation. In addition, the immunogenicity of rodent-based BsAb and especially the relative large size of these molecules (typically 110–150 kDa) are expected to reduce the efficacy for solid tumor therapy.

Antibody engineering by recombinant DNA technology can be exploited to deal with these problems and can be used to rationally design new therapeutic bispecific molecules like the described diabodies (Whitlow *et al.*, 1994; Holliger *et al.*, 1993). Diabodies are small recombinant dimeric antibody fragments formed by cross-pairing of 2 single-chain variable fragment (scFv) molecules. Each chain consists of an immunoglobulin heavy-chain variable domain (VH) connected to an immunoglobulin light-chain variable domain (VL) using a linker that is too short to allow intra-chain pairing between complementary domains. Instead, 2 chains dimerize to form 2 binding sites. Thus, for a bispecific diabody (made from antibodies A and B), the first chain is assembled from the VH domain of antibody A and the VL domain of antibody B; the second chain is assembled from the VH domain of antibody B and the VL domain of antibody A. Co-expression of the 2 chains (VH_A-VL_B, VH_B-VL_A) from a dicistronic expression construct in *Escherichia coli* leads to assembly of bispecific diabodies in the periplasmic space. Unlike other bispecific antibodies, the 2 antigen-binding sites in diabodies are at opposite ends of the molecule and are separated by an average of only 6.5 nm (less than half the distance in immunoglobulin G (IgG); Perisic *et al.*, 1994). Furthermore, the typical m.w. of a diabody is approximately 52 kDa only. Due to this relatively small size, diabodies are expected to show rapid pharma-

cokinetics and improved tumor penetration *in vivo* (Wu *et al.*, 1996).

The murine monoclonal antibody (MAb) MOC-31 recognizes the 38-kDa transmembrane epithelial glycoprotein 2 (de Leij *et al.*, 1994) (EGP2, also known as the CO17-1A antigen or KSA), an antigen that is abundantly expressed on a variety of human carcinomas. Because EGP2 is not shed into the circulation, this glycoprotein is regarded as a suitable target antigen for imaging and immunotherapy of human carcinomas. Several anti-EGP-2 antibodies like 17-1A, KS1/4 and MOC31 have been studied in clinical trials (Riethmüller *et al.*, 1994; Elias *et al.*, 1990; Kosterink *et al.*, 1995). The results of these studies underlined the potential of using antibodies to EGP2 in immunotherapy of human carcinomas. We have screened a series of anti-EGP2 antibodies for their (monovalent) affinity (data not shown). The results showed that MOC31 had the lowest *k*_{off} value, a characteristic that is crucial for tumor retention *in vivo*.

Previously, we studied a hybrid-hybridoma-derived bispecific antibody format of MOC31, (Bis1 = anti-EGP2/anti-CD3), in a phase I clinical study in which Bis1 F(ab')₂ was applied i.v. in renal cell cancer patients receiving interleukin-2 (IL-2) s.c. (Kroesen *et al.*, 1994). The results also indicated the efficacy of this antibody in BsAb-mediated immunotherapy.

Here, we report the construction and characterization of a recombinant bispecific antibody in the diabody format (Dia5v9), which endows activated T cells with EGP2 directed cytolytic potential *in vitro*. This recombinant, small and partially humanized bispecific diabody might have potential in improving the efficacy of BsAb-mediated immunotherapy of human carcinomas *in vivo*.

MATERIAL AND METHODS

Cells

The human small cell lung carcinoma (SCLC) cell lines GLC1 (EGP2⁻) and a clonal derivative thereof, GLC1M13 (EGP2⁺) were maintained in RPMI 1640 (Bio-Whittaker, Verviers, Belgium) based media supplemented with antibiotics and 10% (v/v) FCS (GIBCO, Grand Island, NY) and grown at 37°C in humidified 5% CO₂ atmosphere. COS-7 was maintained in DMEM (Bio-Whittaker), antibiotics and 10% FCS. The murine cytotoxic T cell line CTLL2 was maintained in RPMI, antibiotics and 10% FCS, supplemented with 100 IU/ml human IL-2 (EuroCetus, Amsterdam, The Netherlands). Both COS-7 and CTLL2 were purchased from the ATCC (Rockville, MD).

EGP2 and CD3 transfectants

COS-7 cells were transiently transfected with plasmid CDM8-EGP2 (a kind gift of Dr. A. Linnenbach, Philadelphia, PA) by the calcium phosphate precipitation procedure using the Cellfect transfection kit (Pharmacia, Uppsala, Sweden). Five days after transfection, COS-7 cells were collected after trypsinization and

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used to prepare acetone-fixed cytospin slides. The murine cytotoxic T cell line CTLL2 was transfected with human CD3 ϵ chain using plasmid PSR α -CD3 ϵ (a kind gift of Dr. C. Terhorst, Boston, MA) by electroporation (25 μ g PSR α -CD3 ϵ supercoiled DNA/10⁷ cells in 0.4 cm electrode gap cuvette in 200 μ l medium, 240V, 960 μ F) (Gene Pulser, BioRad, Hercules, CA). Plasmid PSR α -CD3 ϵ contains the neomycin resistance gene, and the obtained murine CTLL2 transfectants stably expressing the human CD3 ϵ chain were selected on culture medium supplemented with G418 (GIBCO) at 1 mg/ml. The resultant cell line CTLL2/humCD3 ϵ was analyzed by flow cytometrical analysis using the FACS-star flow cytometer (Becton Dickinson, Mountain View, CA) using MAb WT32 (see below) and FITC-conjugated rabbit F(ab')₂ anti-mouse Ig (Dakopatts, Glostrup, Denmark) which confirmed membrane expression of CD3 ϵ (data not shown).

Monoclonal antibodies

The MAb MOC-31 (IgG₁, κ) was produced by standard hybridoma technology after immunizing mice with an EGP2-positive SCLC cell line. The MAb MM104 (IgG_{2b}) (a kind gift of Dr. M.J. Mattes, Newark, NJ) recognizes a linear epitope on EGP2 that is clearly distinct (non-overlapping) from the structural epitope recognized by MAb MOC31 (Helfrich *et al.*, 1994b). The MAb 9E10 (IgG₁) hybridoma supernatant was used to detect specific binding of the myc-tagged diabody Dia5v9 in immunohistochemistry and FACS analysis. Anti-(His)₆ tag MAb 13/45/31 (IgG_{2a}), a kind gift from Dianova (Hamburg, Germany), was used to detect (His)₆ tagged fusion proteins. Murine BsMAb Bis1 was purified from the hybrid hybridoma obtained by fusing anti-CD3 hybridoma RIV9 (IgG₃) and anti-EGP2 hybridoma MOC31 (IgG₁) as described previously (Kroesen *et al.*, 1994). The mitogenic anti-CD3 MAb WT-32 was a kind gift of Dr. W. Tax (Nijmegen, The Netherlands). Binding of murine whole antibody was detected using peroxidase-conjugated rabbit anti-mouse Ig (Dakopatts) using 3-amino-9-ethylcarbazole (AEC) as the staining substrate.

Polymerase chain reactions

All polymerase chain reactions (PCR) were carried out in a volume of 50 μ l with 250 μ M dNTPs, 20 pmol of each primer and 0.2 units of proof-read Pwo DNA polymerase (Boehringer Mannheim, Germany), in the manufacturer recommended buffer, using the PE 2400 Thermal Cycler (Perkin Elmer, Norwalk, CT). All PCRs, unless specified otherwise, consisted of an initial 5 min denaturation at 94°C, followed by 25 cycles at 94°C, 60°C (annealing), 72°C (elongation) all for 30 sec, followed by a final 7 min elongation step at 72°C. Sequence integrity of final PCR products was verified using the ALF-express sequencer (Pharmacia) according to standard procedures. All cloning procedures were performed in *E. coli* strain Top10 F' (Invitrogen, Leek, The Netherlands) according to standard procedures.

Diabody format

The format used for this diabody construct (including vector-based features) is essentially as described by McGuinness *et al.* (1996). In short: (5'-3'): RBS-leader1-VH_{CD3v9}-linker1-VL_{MOC31}-stop-RBS-leader2-V H_{MOC31}-linker2-VL_{CD3v9}-Myc-His₆-stop (Fig. 1.V). RBS is the lacZ ribosome binding site CAAGGA. Leaders 1 and 2 are pelB (from pectate lyase gene of *Erwinia carotovora*), and phage fd gene 3 signal sequence, respectively. Linkers 1 and 2 are residues GGGGS and GGGSS, respectively. An outline of the diabody construction procedure is given in Figure 1, I-V.

Anti-EGP2 Fv fragments

Plasmid pCANTABMOC31, containing scFv MOC31, was constructed from the murine hybridoma cell line MOC31 using standard phage display technology (Clackson *et al.*, 1991), with recombinant baculo virus expressed soluble EGP2 (sEGP2) (Helfrich *et al.*, 1994a) as the selective antigen (data not shown). The VL_{MOC31} fragment was amplified from pCANTABMOC31 with primers 1 and 2 (see Fig. 1, I). The sequence of these and following primers are given in Table I. The resulting 366 bp PCR

product was re-amplified with primers 3 (addition of Gly/Ser linker 1 + SacI site) and 4 (encoding 2 in-frame stop codons TAA) (Fig. 1, II), yielding a 461 bp product. Analogously, VH_{MOC31} was amplified with primers 5 and 6 (Fig. 1, I). The resulting 347 bp PCR product was re-amplified with primers 7 [coding RBS (CAAGGA) and leader 2] and 8 (encoding linker 2 + XhoI site) (Fig. 1, II), yielding a 430 bp product. Primers 4 and 7 (and thus PCR products obtained with these primers) overlap partially (Fig. 1, II and III).

PCR-mediated assembly

PCR mediated assembly was performed by SOE-PCR (splicing by overlap extension) (McGuinness *et al.*, 1996; Clackson *et al.*, 1991). In short: Final VL_{MOC31} and VH_{MOC31} PCR products (see above) were assembled by 9 PCR cycles without, followed by 25 cycles with pull-through primers 3 and 8 (Fig. 1, III). The resulting assembled 867 bp PCR product, designated Cassette I, was double-digested with SacI and XhoI (Fig. 1, IV).

Humanized anti-CD3 Fv fragments

Anti-CD3 VH and VL fragments were amplified from plasmid pZZ3 using primers 9/10 and primers 11/12, respectively (Fig. 1.III). pZZ3 (Zhu *et al.*, 1996) (a kind gift of Dr. P. Carter, San Francisco, CA) contains the VH_{CD3v9} and VL_{CD3v9} domains of a humanized anti-CD3_{v9} MAb. Primers 9 and 10 were used to amplify the VH_{CD3v9} sequences from a 0.8 kb MluI restriction fragment of pZZ3, adding in frame a 5'-end SfiI and a 3'-end SacI site to the resulting PCR product. Analogously, primers 11 and 12 were used to amplify VL_{CD3v9}, adding a 5'-end XhoI and a 3'-end NotI site, using the 5.6 kb MluI remnant of pZZ3 as the DNA template (Fig. 1, III). The resulting VH_{CD3v9} (Cassette 2; 412 bp) and VL_{CD3v9} (Cassette 3; 357 bp) PCR products were gel purified and subsequently double digested with the appropriate enzymes (SfiI/SacI and XhoI/NotI, respectively) (Fig. 1, IV).

Cassette cloning

Cassettes 1, 2 and 3, digested with the relevant restriction enzymes, were ligated; the resultant product was subsequently inserted as a SfiI-NotI digested fragment (1531 bp) into the expression plasmid pUC119mycHis6 (a kind gift of Dr. K. FitzGerald, Uxbridge, UK) digested with the same 2 enzymes, yielding the diabody expression plasmid pDia5v9 (Fig. 1.V). In this pUC119 derivative, the diabody Dia5v9 is cloned in frame with an upstream lacZ promotor, pelB signal sequence (L1), and a downstream c-myc-derived sequence (M) allowing detection of the diabody with the 9E10 antibody, as well as a cassette encoding 6 histidine residues (H) permitting IMAC (immobilized metal-ion affinity chromatography) purification. In contrast to plasmid pCANTABMOC31 (Fig. 1, I), pDia5v9 does not contain bacteriophage gene III, which ensures less toxicity and thus a higher yield of antibody product.

Expression and purification Dia5v9 diabody

The Dia5v9 diabody was produced in the *E. coli* nonsuppressor strain SF110F', which is deficient in the proteases DegP and OmpT (Meerman and Georgiou, 1994). One and a half liters of 2 \times TY-AG [2 \times TY; 1.6% (w/v) tryptone; 1% (w/v) yeast extract; 0.5% (w/v) NaCl, supplemented with 100 μ g/ml ampicillin and 2% glucose] were inoculated with 15 ml of an overnight culture of *E. coli* SF110 F' harbouring pDia5v9. Bacteria were subsequently grown at 24°C to an OD₆₀₀ of 0.9. Bacterial cells were spun down (5000 g, 10 min), resuspended in the same volume 2 \times TY-A containing IPTG (isopropyl- β -D-galactopyranoside, Boehringer Mannheim) in a final concentration of 2 mM and grown for 18 hr at 24°C while shaking. Subsequently, bacteria were pelleted again, and an extract was prepared by resuspending the pellet in 50 ml ice cold PBS, 3M NaCl, 1 mM EDTA. After incubation for 30 min on ice, cells and cellular debris were spun down (20,000 g for 1 hr). To the resulting cleared lysate MgCl₂ was added at a final concentration of 10 mM. To reduce viscosity of the lysate, DNaseI (Boehringer Mannheim) was added at a final concentration of 50 μ g/ml with incubation for 1 hr at room temperature (RT). The

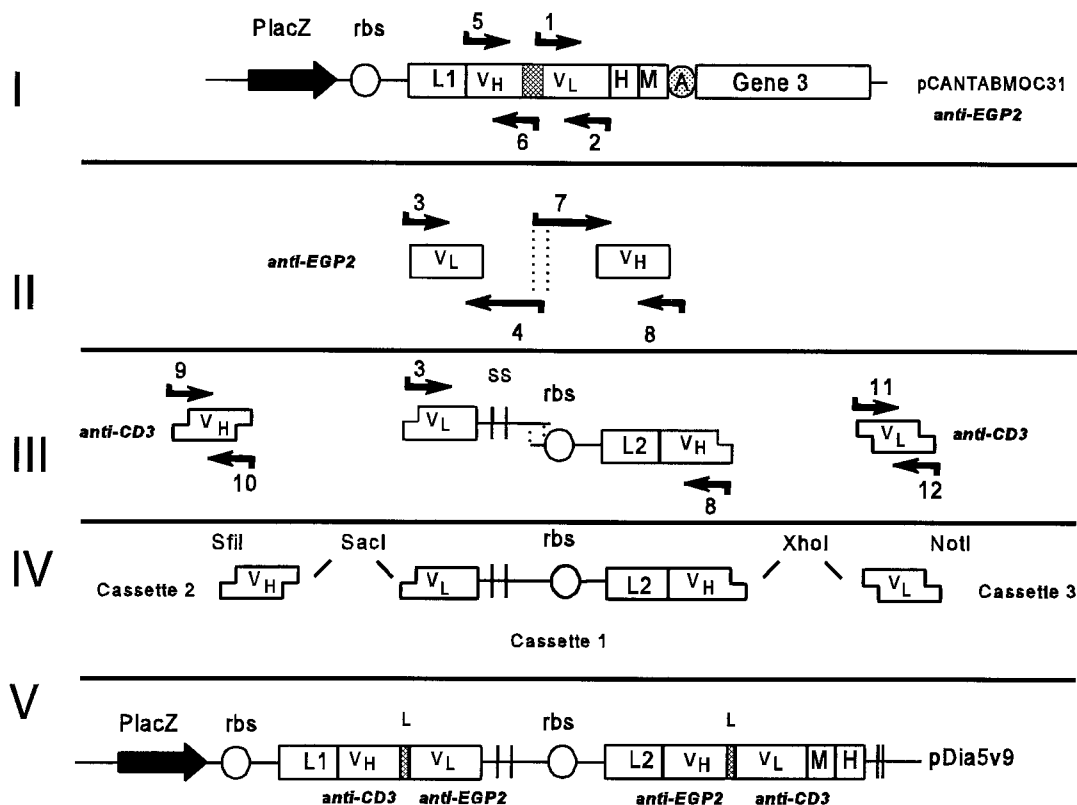


FIGURE 1 – Construction of the bispecific diabody Dia5v9. VH and VL variable regions of the anti-EGP2 scFv MOC31 were PCR amplified from plasmid pCAN-TABMOC31 using primers 5 and 6, and 1 and 2, respectively (I). VL_{MOC31} and VH_{MOC31} PCR products were assembled by SOE-PCR (II) (see Material and Methods) and pull-through primers 3 and 8 (III), yielding Cassette 1, which was double-digested with SacI and XhoI (IV). VH_{anti-CD3} and VL_{anti-CD3} were PCR amplified from the humanized anti-CD3 scFv Ucht1v9 using primers 9 and 10, and 11 and 12, respectively (III). The resultant anti-CD3 PCR products were double digested with SfiI and SacI (for VH_{anti-CD3}), yielding Cassette 2, or XhoI and NotI (for VL_{anti-CD3}), yielding Cassette 3 (IV). Cassettes 1, 2 and 3 were ligated together to form a 5 amino acid linker containing bispecific diabody. The diabody was cloned into a pUC-derived expression vector under the control of the lacZ promoter (PlacZ), with lacZ ribosome binding sites (rbs) and the pelB leader sequence (L1) and the phage fd gene 3 leader (L2) for secretion and subsequent assembly of the diabody in the periplasmic space (V). Locations of the used PCR primers 1–12 are indicated by small arrows. S, stop codon; A, amber mutation; H, 6 × His tag; M, Myc tag.

diabody was further released from the bacterial cell pellet by 2 consecutive freeze/thaw cycles and by partial digestion of the cell wall with lysozyme (0.6 mg/ml) (Boehringer Mannheim). The cleared lysates were combined and were filter sterilized (0.22 µm) before subjecting it to IMAC. IMAC purification of recombinant diabody was performed under non-denaturing conditions using a 1 ml Ni²⁺NTA-silica column essentially according to the manufacturer's (Qiagen, Hilden, Germany) recommendations. The column was washed with 20 column volumes of 20 mM sodium phosphate (NaP: NaH₂PO₄/Na₂HPO₄) buffer, 3.0 M NaCl, pH 8.0, followed by 10 column volumes 20 mM NaP, 0.5 M NaCl, 20 mM imidazole, pH 7.0. Elution was with 20 mM NaP, 0.5 M NaCl, 300 mM imidazole, pH 7.0. The eluted diabody fraction was dialyzed against an excess of PBS using Slide-A-Lyzer dialysis cassettes with a MW cutoff of 10 kDa (Pierce, Rockford, IL).

SDS-PAGE

Approximately 5 µg of IMAC purified Dia5v9 were dissolved in SDS-PAGE sample buffer with 5% 2-mercaptoethanol (2-ME) and heated at 100°C for 3 min. SDS-PAGE was performed on 10% polyacrylamide gels. (Mini-Protein II, BioRad). The gel was stained with sypro-orange (BioRad) protein staining and was visualized by UV transillumination.

Size-exclusion FPLC

The aggregation state of the IMAC-purified Dia5v9 was assessed by size-exclusion FPLC on a Superose 6 (Pharmacia)

column in PBS, pH 7.4, at a flow rate of 0.5 ml/min. The column was equilibrated with a set of standard proteins (BSA dimer 136 kDa, BSA monomer 68 kDa, ovalbumin 43 kDa, trypsin inhibitor 20 kDa).

Affinity of target cell binding by diabody Dia5v9

Kinetic measurement by SPR was performed in the BIAcore2000 (Pharmacia). Recombinant soluble EGP2, expressed in baculovirus (Helfrich *et al.*, 1994a), was covalently coupled to a CM-5 sensorchip (Pharmacia) via free amine chemistry, resulting in a surface of 500 resonance units (RU). This low density surface was saturated with a Dia5v9 solution using a flow rate of 20 µl/min. Dissociation rates were then calculated using the BIAevaluation software (Pharmacia) from the sensorgrams. The off-rate was determined by curve fitting.

Stability of diabody

Aliquots of the diabody Dia5v9 were stored for more than 6 months in PBS at 4°C or at –80°C and were compared with a freshly prepared Dia5v9 sample. To assess the stability in human serum, 20 µl of the diabody and 100 µl serum from a healthy donor were incubated at 37°C for different periods. The samples were subsequently titrated on COS7 cells transfected with plasmid CDM8-EGP2.

TABLE I – OLIGONUCLEOTIDES USED FOR DIABODY CONSTRUCTION¹

Primer 1:	GAC ATT GTG CTR ACC CAR TCT CCA
Primer 2:	CGT GAC AAC ATA TGG CGC GCC TTA TTA CCG TTT GAT TTC CAG CTT GGT GCC
Primer 3:	GGC CAA GGT ACC ACG GTC ACC GTG AGC TCA GGT GGA GGC GGT TCA GAC ATT GTG CTR ACC CAR TCT CCA
	<u>SacI</u>
Primer 4:	GGC ACC ACG AGT GGT ATG GCA AAG AGG AGT TTT TTC ACT GAC TGT CTC CTC GTG ACA ACA TAT GGC GCG
	CCT TAT TAC CGT TT
Primer 5:	AGG TSM ARC TGC AGS AGT CWG G
Primer 6:	TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CC
Primer 7:	CTT TGC CAT ACC ACT CGT GGT GCC ATT TTA CTC CGC GGC TGC CCA ACC AGC GAT GGC CSA GGT SMA RCT
	GCA GSA GTC WGG
Primer 8:	CAC AAT GTC GCT CGA GCC TCC ACC TGA GGA GAC AGT GAC TGT GGT ACC TTG GCC
	<u>XhoI</u>
Primer 9:	CAT GCC ATG ACT CGC <u>GGC CCA GCC GGC</u> CAT GGC CGA GGT TCA GCT GGT GGA GTC TGG
	<u>SfiI</u>
Primer 10:	GGT AGC TAG CTC ATG <u>AGC TCA</u> CGG TGA CCA GGG TTC CTT GAC C
	<u>SacI</u>
Primer 11:	GGT GGA GGC TCG <u>AGC</u> GAT ATC CAG ATG ACC CAG TCC CCG
	<u>XhoI</u>
Primer 12:	GAG TCA TTC <u>TGC GGC CGC</u> CCG TTT GAT CTC CAC CTT GGT GCC
	<u>NotI</u>

¹All primers are shown 5' → 3' and are numbered for simplicity. The sequences are given using the IUPAC nomenclature of mixed bases (shown in bold capital letters, R = A or G; S = C or G; M = A or C; W = A or T). Restriction site sequences are underlined.

Analysis of bispecific diabody binding by immunohistochemistry

All incubations were for 30 min at RT, each followed by 3 consecutive washing steps with PBS. Bispecific binding of diabody Dia5v9 to both EGP2 and human T cells was assessed by "capture" immunohistochemistry. The procedure is schematically represented in Figure 4. In short, acetone fixed cryo-sections of human tonsils were incubated with 1 µg IMAC-purified Dia5v9 in PBS. After washing with PBS, the sections were incubated with 10 µg recombinant EGP2 (sEGP2) in TC100 medium (GIBCO) with 10% v/v FCS. sEGP2 is a recombinant truncated form of EGP2 that only comprises the extracellular domain of EGP2 (Helfrich *et al.*, 1994a). The presence of diabody-captured sEGP2 was detected by incubation with the anti-EGP2 MAb MM104 and subsequently visualized with horseradish peroxidase-conjugated goat F(ab')₂ anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) using AEC as the staining substrate. In negative control experiments, the diabody Dia5v9, the capture antigen sEGP2, or MAb MM104 was excluded from the incubation scheme. Direct binding of Dia5v9 to T cells was demonstrated by normal immunohistochemical analysis using anti-myc tag MAb 9E10 and peroxidase-conjugated rabbit anti-mouse Ig.

CTL preparation

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood of healthy volunteers. Isolation was performed by density centrifugation of diluted (1 to 1 in PBS) blood on Lymphoprep (Nycomed, Oslo, Norway) at 800 g for 20 min. The PBMC fraction was washed twice by resuspension in RPMI-1640 and centrifugation at 500 g (first time) and 300 g (second time) for 10 min. After isolation, PBMC were collected in complete medium consisting of RPMI-1640 supplemented with 2% heat inactivated human pooled serum, 2 mM glutamine and 60 µg/ml gentamicin. The cytotoxic T lymphocyte (CTL) effector cells were prepared by *in vitro* incubation of PBMC for 3 days in complete medium supplemented with 5% (giving about 0.5 µg IgG/ml, final concentration) culture supernatant of the mitogenic anti-CD3 MAb WT-32, followed by washing and incubation for 2 additional days in complete medium supplemented with 100 IU/ml IL-2 (EuroCetus, Amsterdam, The Netherlands).

⁵¹Cr-release assay

⁵¹Cr-release assays were performed according to standard procedures to assess diabody redirected T-cell cytotoxicity. All determi-

nations were done in triplicate in the presence of 100 IU/ml IL-2. Prior to the assay, 5×10^6 EGP2⁺ (GLC1M13) or EGP2⁻ (GLC1) target cells were suspended in 100 µl culture medium containing 3.7 MBq Na₂⁵¹CrO₄ (Amersham, Aylesbury, UK) and incubated for 1 hr at 37°C in a humidified, 5% CO₂ containing atmosphere. Unbound Na₂⁵¹CrO₄ was removed by washing the cells 3 times with culture medium. Aliquots of 100 µl medium containing 2.5×10^3 ⁵¹Cr-labelled target cells were pipetted into each well of a 96-well round bottom microtiter plate. Subsequently, 50 µl medium containing various amounts of either Bis1 or diabody Dia5v9 and 50 µl CTL effector cells were added to each well to give the desired final Bis1 or Dia5v9 concentration (10 nM, 1 nM, 0.1 nM) and effector to target ratio (E:T = 100, 10, 1) in a final volume of 200 µl per well. The microtiter plates were centrifuged at 50 g for 2 min and incubated at 37°C, 5% CO₂ for 4 hrs. After incubation, the plates were centrifuged at 200 g for 5 min, and 100-µl samples taken from the supernatant were counted in a gamma counter (LKB Pharmacia, Bromma, Sweden) for 5 min. Cell lysis was calculated from the percentage ⁵¹Cr released, according to the following formula:

$$(\text{exp. release} - \text{spont. release}) / (\text{max. release} - \text{spont. release}) \times 100\%$$

Maximal release was determined from a sample to which 100 µl 2% Triton X-100 solution was added instead of BIS-1 or Dia5v9 and effector cells. Spontaneous release was determined from a sample to which 50 µl medium was added instead of effector cells.

RESULTS

Vector design for diabody expression

The plasmid pDia5v9 was constructed to express the anti-CD3/anti-EGP2 diabody by co-secretion of the 2 component cross-over scFv fragments from a synthetic di-cistronic operon cloned between the HindIII and EcoRI sites of pUC119mycHIS6. In diabody Dia5v9, the C-terminus of the anti-CD3_{v9} VH domain was first connected to the N-terminus of the anti-EGP2 VL domain using a 5 amino acid linker nr. 1 (GGGGS) to restrict intra-chain pairing of VH and VL. A second cross-over scFv was then constructed by connecting the C-terminus of the anti-EGP2 VH domain to the N-terminus of the anti-CD3_{v9} VL domain using a similar linker nr. 2 (GGGSS). Both the anti-CD3_{v9} VH and anti-CD3_{v9} VL domains were successfully amplified from MluI fragments derived from

plasmid pZZ3, containing the humanized anti-CD3 scFv HuMabUcht 1v9.

The diabody was assembled using SOE-PCR and PCR cassette ligation cloning. To minimize PCR errors, polymerase Pwo with proof-reading capacity was used. Indeed, none of the generated PCR products showed any nucleotide misincorporation in sequence analysis (data not shown).

Diabody expression and purification

The anti-EGP2/anti-CD3 diabody was secreted from *E. coli* strain SF110F' containing the plasmid pDia5v9 grown in a volume of 1.5 l in a 5-l shaker flask. The diabody was purified by IMAC from lysed bacterial pellets and recovered with a typical yield of 200 µg/l of culture. The eluted diabody fraction was judged to be >90% pure.

SDS-PAGE analysis of diabody Dia5v9

The purified diabody Dia5v9 was analyzed by SDS-PAGE under reducing conditions (Fig. 2). The 2 component chains of the diabody (calculated m.w. 28,416 Da and 30,279 Da, respectively) were not resolved under the electrophoretic conditions used. The diabody showed an apparent m.w. of approximately 30 kDa and was judged to be >90% pure.

Size-exclusion FPLC

The solution behaviour of diabody Dia5v9 was analyzed by size exclusion FPLC that revealed a major peak at a retention time of 26.2 min. Samples taken from this peak confirmed the presence of the diabody using immunocytochemistry on COS7 cells transfected with EGP2 (data not shown). The diabody is likely a dimer of M_r approx. 50 as judged by its retention time when compared with the standard protein m.w. markers (136 kDa, 23.2 min; 68 kDa, 25.5 min; 42 kDa, 26.8 min; 20 kDa, 29 min) (Fig. 3). Although Dia5v9 yielded predominantly dimer, small amounts of higher molecular mass components were observed. However, immunoreactivity was only observed in samples of the major peak (data not shown).

Specificity of binding

The specific binding of Dia5v9 to CD3 was demonstrated using the human CD3ε chain transfectant murine cell line CTLL2/humCD3ε in flow cytometrical analysis. There was a clear shift in fluorescence intensity for Dia5v9 compared with the non-transfected parental cell line. Anti-EGP2 MAb MOC31 showed no binding to CTLL2/humCD3ε. By immunocytochemistry, Dia5v9



FIGURE 2 – SDS-PAGE analysis of IMAC purified diabody Dia5v9. Dia5v9 was produced in IPTG induced *E. coli* SF110 F' harboring plasmid pDia5v9. IMAC purification of diabody Dia5v9 was performed under non-denaturing conditions using on Ni^{2+} NTA-silica. The 2 component chains of the diabody (calculated m.w. 28,416 Da and 30,279 Da, respectively) were not resolved under the electrophoretic conditions used. The purified diabody showed a single protein band with an apparent m.w. of approximately 30 kDa. The relative position of the m.w. markers are indicated by dashes on the left.

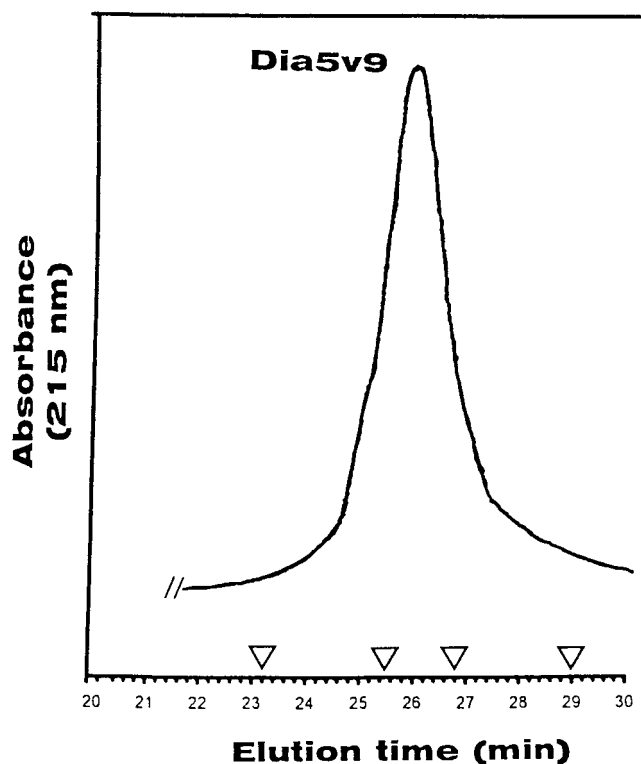


FIGURE 3 – Size-exclusion FPLC profile of IMAC-purified diabody Dia5v9 on a calibrated Superose 6 column eluted with PBS, pH 7.4, and run at a flow rate of 0.5 ml/min. Under these conditions the retention times of the m.w. standards are as follows: bovine serum albumin (BSA) dimer, 136 kDa, 23.2 min; BSA monomer, 68 kDa, 25.5 min; ovalbumin, 42 kDa, 26.8 min; and trypsin inhibitor, 20 kDa, 29 min. The relative positions of these standard proteins are indicated by open triangles.

showed strong and specific binding to EGP2 transfected COS7 cells (data not shown).

Affinity of target cell binding by diabody Dia5v9

The binding kinetics of diabody Dia5v9 for EGP2 was determined by surface plasmon resonance using the BIAcore apparatus. The diabody showed an off-rate of $7.27 \pm 0.04 \times 10^{-4} \text{ sec}^{-1}$, which was approx. 2-fold faster than that of the parental anti-EGP2 scFv ($3.4 \times 10^{-4} \text{ sec}^{-1}$).

Stability of diabody

The stability of diabody Dia5v9 in PBS appeared to be very high. Storage of Dia5v9 in PBS at 4°C for more than 6 months did not result in a significant decrease of immunoreactivity. However, when the diabody stability in human serum at 37°C was assessed, a decrease of 50% in immunoreactivity was observed after 12 hr of incubation (data not shown).

Analysis of bispecific binding by immunohistochemistry (Fig. 4)

Simultaneous binding capacity of diabody Dia5v9 to both antigens (EGP2 and CD3) was investigated by "capture" immunohistochemistry. In this procedure, the anti-CD3 specificity of Dia5v9 is responsible for binding of the diabody to CD3⁺ T cells present in a cryostat section of a human tonsil. The anti-EGP2 specificity of the CD3 bound diabody was subsequently evaluated by its capability to capture sEGP2 from a solution applied to this section. Presence of diabody-captured sEGP2 was demonstrated using a MAb (MM104) that reacts with the captured EGP2 through an other non-overlapping epitope. A representative tonsil section stained by this procedure is shown in Figure 5a, b. T-cell areas (T)

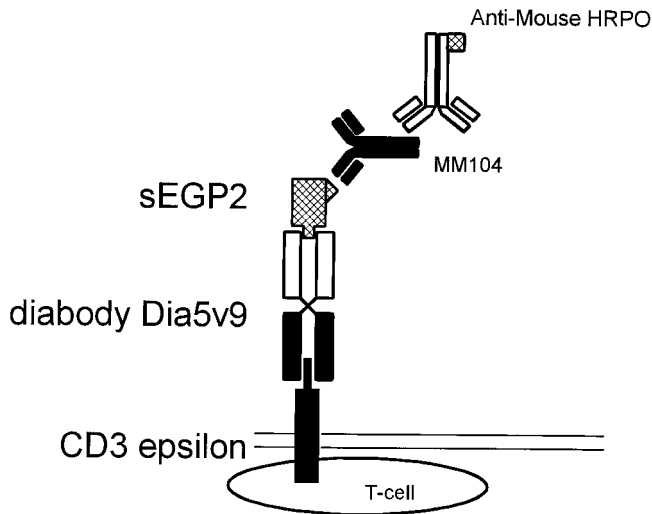


FIGURE 4 – Schematic presentation of “capture” immunohistochemistry for evaluation of the bispecific binding capacity of diabody Dia5v9. The anti-CD3 specificity of Dia5v9 is responsible for binding to the CD3 ϵ chain on T cells in a cryostat section of human tonsil tissue. The anti-EGP2 specificity of the bound diabody captures soluble sEGP2 that is subsequently applied to this section. The presence of diabody-capture sEGP2 is subsequently demonstrated using an anti-EGP2 MAb (MM104) that reacts with the captured sEGP2 through an other epitope followed by anti-mouse HRPO-based immunohistochemical staining (molecules in the diagram are not to scale).

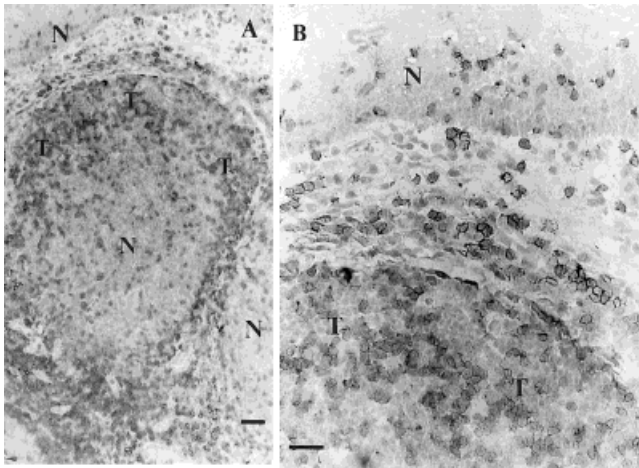


FIGURE 5 – Immunohistochemical analysis of bispecific diabody binding. Bispecific binding capacity of Dia5v9 was assessed by “capture” immunohistochemistry as explained in Material and Methods and outlined in Figure 4. A representative tonsil section stained by this procedure is shown. T-cell areas (T) are strongly stained, whereas non-T-cell areas (N) are negative (scale bars: 50 μ m).

are strongly stained, whereas non-T-cell (N) areas were negative. In control experiments in which the diabody Dia5v9, the capture antigen sEGP2, or MAb MM104 were excluded from the incubation scheme, no staining was observed (data not shown). Staining of a tonsil section with an anti-CD3 MAb (RIV-9) using standard immunohistochemistry gave a similar T-cell specific staining pattern (data not shown).

Re-directed T-cell cytotoxicity

The efficacy of diabody Dia5v9 in directing activated T cells to specifically lyse EGP2 expressing SCLC tumor cells (GLC1M13)

is shown in Figure 6. A similar potency was seen for the diabody Dia5v9 and BsMAb Bis1 (F(ab')₂) in mediating tumor cell lysis over the range of effector to target cell ratios tested (1:1, 10:1 and 100:1) at the different antibody concentrations (0.1 nM, 1 nM, 10 nM) tested (Fig. 7). At an antibody concentration of 10 nM and an effector to target ratio of 100, up to 47% lysis was observed for Dia5v9. Under the same conditions, BsMAb Bis1 (F(ab')₂) showed a cell lysis of 65%. An identical background lysis of <10% was observed for both diabody Dia5v9 and BsMAb Bis1 (F(ab')₂) when EGP2⁻ target tumor cells (SCLC cell line GLC1) were used in the assay (Figs. 6, 7).

DISCUSSION

Bispecific antibodies with specificity for a tumor-associated antigen on the target cell and the CD3 triggering molecule on the T cell are capable of redirecting cellular cytotoxicity toward tumor cells both *in vitro* and *in vivo*. Feasibility and effectiveness of this

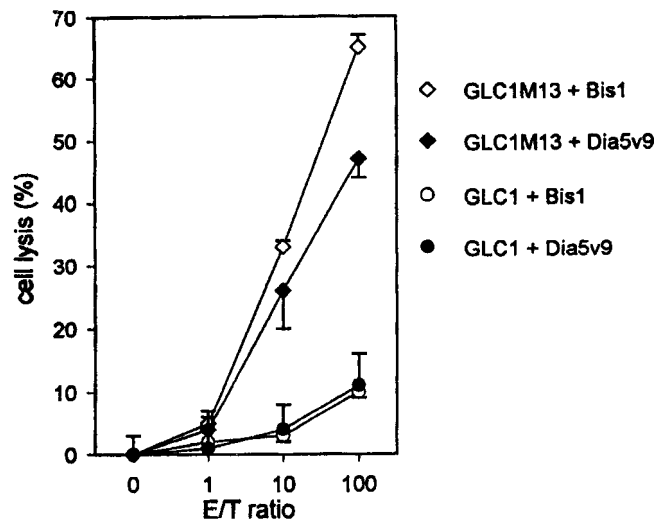


FIGURE 6 – *In vitro* cytolytic activity of activated T lymphocytes against EGP2⁺ GLC1M13 target cells (filled and open diamonds) and EGP2⁻ GLC1 target cells (filled and open circles) induced by 10 nM Bis1 F(ab')₂ (open diamonds and open circles) and 10 nM diabody Dia5v9 (filled diamonds and open circles) at an effector to target ratio of 100. Redirected T-cell mediated cytotoxicity was assessed in a standard 4 hr ⁵¹Cr-release assay.

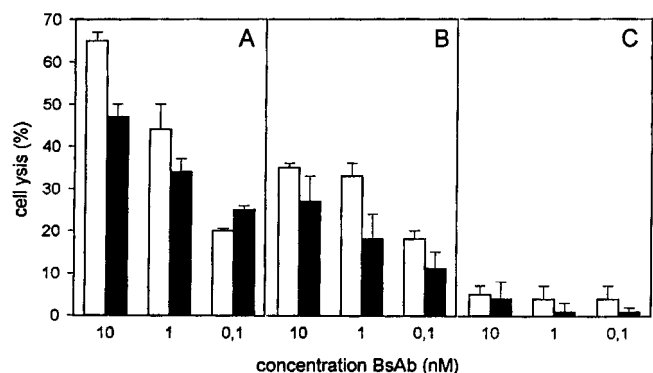


FIGURE 7 – Lysis of GLC1M13 lung carcinoma cells mediated by anti-EGP2/anti-CD3 diabody Dia5v9 (black bars) or BsMAb Bis1 (F(ab')₂) (white bars) at different E:T ratios and Ab concentrations. Effector cell to target cell ratios are: (A) 100, (B) 10 and (C) 1. Data shown are the means (\pm SD) of triplicate measurements.

immunotherapeutical concept has been studied extensively in pre-clinical models and in phase I clinical trials (reviewed in Bolhuis *et al.*, 1996). BsMAbs have traditionally been prepared by chemically linking 2 different MAbs or by hybrid-hybridoma technology. Both of these approaches present challenges with respect to yield, purity and immunogenicity of the formed antibody. It is evident that the production of large amounts of recombinant clinical grade bispecific antibodies would greatly facilitate their use in clinical settings.

Recombinant DNA technology has been used to manipulate the size and shape of the BsMAb in the construction of scFv based bispecific diabody (Zhu *et al.*, 1996; Holliger *et al.*, 1996). Diabodies are a new class of dimeric antibody fragments that can be engineered to harbour 2 different antigen-binding sites that are at opposite ends of the molecule. Diabodies are similar in size to Fab' fragments (52 kDa), which should facilitate penetration of tumors and clearance from the serum (Wu *et al.*, 1996).

Diabodies can be expressed by secretion from bacterial cultures; this is in contrast to the single-chain bispecific scFv fragments that generally must be re-folded from inclusion bodies. The construction, production in mammalian CHO cells and characterization of a functional murine bispecific scFv [(scFv)₂] of the same specificities, derived from other hybridomas, was previously published by Mack *et al.* (1995).

For the generation of a bispecific diabody suitable for human carcinoma therapy, we have aimed to construct a small and partially humanized recombinant molecule with dual specificity for both a well-established pan-carcinoma associated target antigen, EGP2 (alias CO17-1A antigen), and the signal transducing CD3 ϵ chain of TCR/CD3 complex. In this bispecific diabody we used the anti-EGP2 scFv MOC31, derived from its corresponding murine hybridoma. Using surface plasmon resonance (SPR) with BIAcore technology, we have previously compared the kinetics of binding of a panel of anti-EGP2 antibodies. This analysis showed that the scFv MOC31 antibody had the slowest off-rate ($3 \times 10^{-4} \text{ sec}^{-1}$) (data not shown), a characteristic that is crucial for tumor retention *in vivo*. The binding kinetics of diabody Dia5v9 for the target cell antigen EGP2 showed an off-rate of $7.27 \pm 0.04 \times 10^{-4} \text{ sec}^{-1}$, which was approximately 2-fold faster than that of the parental scFv MOC31 ($3.4 \times 10^{-4} \text{ sec}^{-1}$). This result indicates that the anti-EGP2 binding site in the Dia5v9 diabody format is slightly impaired.

The *a priori* co-secretion of 2 cross-over scFv fragments may give rise to 2 types of dimer: active heterodimers and likely inactive homodimers. We assessed what fraction of IMAC purified Dia5v9 is present as functional bispecific diabody by titration of the diabody in both "capture" immunohistochemistry and "normal" immunochemistry on human tonsil cryostat sections. In "normal" immunochemistry, specific binding of diabody dimers to T cells is detected; in "capture" immunohistochemistry, T cells will only be stained after binding of functional diabody that is capable of also binding to the sEGP2 capture antigen. The results showed no significant differences in the diabody titers, indicating that the IMAC isolated dimers are largely functional heterodimers. This suggests that pairing of cognate VL and VH domains is significantly preferred over non-cognate pairings, because equal expression of each chain plus random pairing is expected to yield only 50% of the active heterodimer and 25% of each of the inactive homodimers. Our results compare favorably with the data obtained for the humanized anti-p185HER2/anti-CD3 diabody described by Zhu *et al.* (1996). Their results show that approx. 75% of the protein A-purified anti-p185HER2/anti-CD3 diabody dimer is capable of binding p185HER2 as shown by antigen titration. The anti-CD3 and anti-Her specificities of this humanized diabody were both grafted on the human 4D5 human framework. The use of the same framework residues in both specificities might explain the formation of approx. 25% inactive homodimers. Furthermore, the use of protein A chromatography isolates all possible heterodimers and homodimers with equal efficiency. The diabody Dia5v9

described in this work is constructed using 2 very different variable domain frameworks (the human 4D5 framework for anti-CD3 and a murine framework for anti-EGP2); this might explain the reduced formation of non-functional homodimers. Furthermore, diabody Dia5v9 was isolated by IMAC, which purifies only dimers containing a polyhistidine tag, thus active heterodimers and the likely inactive homodimers derived of the second cistron (Fig. 1V). The later homodimers would contain 2 His-tags. In IMAC, these double-tagged homodimers would be detected as a second discrete protein peak upon elution with a linear imidazole gradient. When performing this experiment, we did not observe such a second diabody protein peak (data not shown).

The solution behavior of Dia5v9 was analyzed by size-exclusion FPLC. This experiment confirmed our conclusion that Dia5v9 is most likely a dimer of approx. 50 kDa. Diabodies are rather compact in structure, which might explain why Dia5v9 elutes slightly faster than its calculated m.w. would predict. Although Dia5v9 yielded predominantly dimer, small amounts of higher molecular mass components could be observed. However, immuno-reactivity was only detected in samples taken from the major peak (data not shown).

The bispecific diabody Dia5v9, expressed in *E. coli* and purified in one step by IMAC, was able to induce preactivated cytotoxic T cells to kill the target cells (EGP2⁺ SCLC cell line) efficiently. The potency of the diabody in the cytotoxicity assay appears to be comparable with an analogous hybrid-hybridoma bispecific antibody Bis1 that employs another anti-CD3 antibody (RIV9) as the effector specificity. Holliger *et al.* (1996) observed an increased potency of their bispecific diabody in *in vitro* CD3 retargeted cytotoxicity using an idiotypic marker on a mouse B-cell lymphoma when compared to an analogous hybrid-hybridoma bispecific antibody. These researchers suggest that in bispecific diabody mediated cytotoxicity, the distance (6.5 nm) between target and cytotoxic cells resembles the distance of the natural interaction of the antigen-presenting MHC molecules on target cells with the specific T-cell receptors of the effector cells. A tighter effector-target cell interface may thus lead to a higher local concentration of the cytotoxic cocktail.

To reduce the size of the diabody further and thereby increase its rigidity, the 5 amino acid linker between VH and VL was omitted by connecting the VH and VL domains of each chain directly, yielding the zero-linker diabody Dia0v9. However, the zero-linker diabody Dia0v9 showed no immunoreactivity (data not shown). Using molecular modelling, Holliger *et al.* (1993) have analyzed the effects of joining the C terminus of the VH domain directly to the N terminus of the VL domain. The analysis suggests that the use of a zero-linker might result in possible clashes (*e.g.*, between 2 VH domains as they pack together). These workers proposed that forced packing of the domains can indeed lead to structural alterations at the antigen-binding site. These structural alterations might induce complete loss of both binding affinities as is observed for our zero-linker diabody. Indeed, studies with individual bispecific diabodies (Holliger *et al.*, 1993) and diabody repertoires (McGuinness *et al.*, 1996) indicate that active zero-linker diabodies may only be created for a subset of Fv combinations. Therefore, the 5-residue format appears to be the format of choice for most diabodies.

When the anti-CD3v9 VH and VL domains of Dia5v9 were replaced by the VH and VL domains of the well-established anti-CD3 scFv OKT3, no cytotoxic capacity was observed in redirected T-cell mediated killing of EGP2⁺ cell lines. Furthermore, the resulting diabody (designated Dia5okt3) showed no anti-CD3 reactivity with the CD3 ϵ chain transfectant cell line CTLL2/humCD3 ϵ . A rough comparison of IC50 obtained using scFv-OKT3 (Kiprianov *et al.*, 1997) with that of Fab-UCHT1v9 (Zhu and Carter, 1995) shows an approx. 100-fold difference in affinity. Therefore, it is plausible that a relative low yield of Dia5okt3 combined with the low affinity of its anti-CD3 domain resulted in inability to detect CD3 binding. The anti-EGP2

reactivity toward EGP2 transfectant COS-7 cells of diabody Dia5okt3 was fully retained (data not shown).

Diabody Dia5v9 is produced in an amount of 200 µg/l, which is similar to the expression amount of MOC31 scFv (data not shown). Production amounts may be improved by high-cell-density fermentation in *E. coli* using an optimized expression vector, improving folding in a bacterial environment (Knappik and Plückthun, 1995), or high level expression in the yeast *Pichia pastoris* (Fitzgerald *et al.*, 1997).

Diabody Dia5v9 stability in PBS appeared to be very high with no significant reduction in immunoreactivity after more than 6 months' storage at 4°C. Moreover, the diabody immunoreactivity was resistant to multiple freeze/thaw cycles. Apparently, the immunoreactivity of the component cross-over scFvs in this diabody format is rather stable, because most antibodies in the traditional scFv format (*e.g.*, scFv MOC31) lose their immunoreactivity under these conditions (data not shown). Stability in human serum at 37°C appeared to be low (half-life approx. 12 hr). The stability of Dia5v9 may be improved further by the provision of 1

or 2 disulfide bridges between the VH-VL pairs (Fitzgerald *et al.*, 1997).

Bispecific diabodies are an attractive new class of bispecific antibody fragments that in theory are relatively simple to construct. However, there are only few examples of biologically functional bispecific diabodies potentially suitable for T-cell retargeted tumor therapy in human carcinoma (Zhu *et al.*, 1996). The Dia5v9 diabody described here is an example of a biologically active and partially humanized T-cell redirecting bispecific diabody directed against the very well-established carcinoma target antigen EGP2 (CO17-1A antigen). This diabody may prove to be potent in retargeting activated T lymphocytes to lyse various human carcinomas *in vivo*.

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