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 pancarcinoma associated target antigen EGP2 (epithelial glycoprotein 2, also known as the CO17-1A antigen or KSA) and the CD3ε chain of human TCR/CD3 complex. The murine anti-EGP2 (MOC31) single chain variable fragment (scFv) and the humanized anti-CD3 (Dia5v9) scFv were cast into a diabody format (db) using a short 5 amino acid Gly-Ser linker between immunoglobulin heavy-chain and light-chain variable domains. Purification of the polystatine tagged Dia5v9 was achieved from extracts of protease deficient E. coli by IMAC chromatography. The Dia5v9 diabody showed strong binding to EGP2 and CD3 in transfected cells. The in vitro efficacy ofDia5v9 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 ^{51}Cr-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., 1993) 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 (VHA\_VLB\_VHB\_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); Persic 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 pharmacokinetics 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, KSI14 and MOC31 have been studied in clinical trials (Riehmüller 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 tumor retention (monovalent) affinity (data not shown). The results showed that MOC31 had the lowest k_{el} value, a characteristic that is crucial for tumor retention in vivo.

Previously, we studied a hybrid-hybridoma-derived bispecific antibody format of MOC31, (BisI = anti-EGP2/anti-CD3), in a phase I clinical study in which BisI 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\_1) and a clonal derivative thereof, GLC1M13 (EGP2\_2) 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% CO_{2} 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 CTL2 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 Calciumfect 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 CD3ε chain using plasmid PSRo-CD3ε (a kind gift of Dr. C. Terhorst, Boston, MA) by electroporation (25 µg PSRo-CD3ε supercoiled DNA/10^7 cells in 0.4 cm electrode gap cuvette in 200 µl medium, 240V, 960 µF) (Gene Pulser, BioRad, Hercules, CA). Plasmid PSRo-CD3ε contains the neomyzin resistance gene, and the obtained murine CTLL2 transfectants stably expressing the human CD3ε chain were selected on culture medium supplemented with G418 (GIBCO) at 1 mg/ml. The resultant cell line CTLL2/humCD3 was analyzed by flow cytometrical analysis using the FACS-star flow cytometer (Becton Dickinson, Mountain View, CA) using MAb WT32 (see Fig. 1, I). The binding of the myc-tagged diabody Dia5v9 in immunohistochemistry (Fig. 1, I, II) and FACS analysis. Anti-(His)6 tag MAb 13/45/31 (IgG2a), a kind gift of Dr. W. Tax (Nijmegen, The Netherlands), was used to detect specific expression of CD3ε chain (data not shown).

Monoclonal antibodies

The MAb MOC-31 (IgG1,κ) was produced by standard hybridoma technology after immunizing mice with an EG2-positive SCLC cell line. The MAB MM1014 (IgG2a) (a kind gift of Dr. M.J. Mattes, Newark, NJ) recognizes a linear epitope on EG2 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 (IgG2a), a kind gift from Dianova (Hamburg, Germany), was used to detect (His)6 tagged fusion proteins. Murine BSMAB B1s1 was purified from the hybrid hybridoma obtained by fusing anti-CD3 hybridoma with Plasmid pCANTABMOC31 (data not shown).

Polymerase chain reactions

All polymerase chain reactions (PCR) were carried out in a volume of 50 µl with 250 µM 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-VHCD3ΔpVH-linker1-VLMOCC1-stop-RBS-leader2-VHMOCC1-linker2-VLMOCC1-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 P. fluorescens) and phage tfd gene 3 signal sequence, respectively. Linkers 1 and 2 are residues GGGGS and GGGSSS, 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., 1994b) 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 1, was double-digested with ScaI 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) is a kind gift of Dr. P. Carter, San Francisco, CA, contains the VHCD3 and VLCD3 domains of a humanized anti-CD33 MAb. Primers 9 and 10 were used to amplify the VHCD3 fragment 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 VLCD3, adding a 5'-end XhoI and a 3'-end NotI site, using the 5.6 kb MluI fragment of pZZ3 (as the DNA template (Fig. 1, III). The resulting VHCD3 (Cassette 2; 412 bp) and VLCD3 (Cassette 3; 357 bp) PCR products were gel purified and subsequently double digested with the appropriate enzymes (SfiI/ScaI and XhoI/NotI, respectively) (Fig. 1, IV).

Cassette cloning

Cassettes 1 and 2, 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 into frame with an upstream lacZ promoter, 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 bacteria-phage 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 × TY-AG [2 × TY; 1.6% (w/v) tryptone; 1% (w/v) yeast extract; 0.5% (w/v) NaCl, supplemented with 100 µg/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 OD560 of 0.9. Bacterial cells were spun down (5000 g, 10 min), resuspended in the same volume 2 × 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 µg/ml with incubation for 1 hr at room temperature (RT). The
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 Ni\(^2\)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: NaH\(_2\)PO\(_4\)/Na\(_2\)HPO\(_4\) ) 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-Protean 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., 1994), 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 CDMS-EGP2.

**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), VH\(_{\text{MOG}}\) and VH\(_{\text{MOC31}}\), 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\(_{\text{anti-CD3}}\) and VL\(_{\text{anti-CD3}}\) 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 SacI and SacI (for VH\(_{\text{anti-CD3}}\)), yielding Cassette 2, or XhoI and NotI (for VL\(_{\text{anti-CD3}}\)), yielding Cassette 3 (IV). Cassette 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 pEB 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\(^{\text{His}}\); M, Myc tag.
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 containing various amounts of either Bis1 or diabody Dia5v9 and 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 MB 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 (Nycos, 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 determinations were done in triplicate in the presence of 100 IU/ml IL-2. Prior to the assay, 5 × 105 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 × 105 Cr-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:

\[ \text{% Release} = \frac{\text{exp. release} - \text{spont. release}}{\text{max. release} - \text{spont. release}} \times 100\% \]

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-CD3α VH domain was first connected to the N-terminus of the anti-EGP2 VL domain using a 5' linker

\[ \text{Primer 1: GAC ATT GTGCTR ACC CAR TCT CCA} \]
\[ \text{Primer 2: CGT GAC AAC ATA TGG CCG GCC TTA TTA CCG TTT GAT TTC CAG CTT GGT GCC} \]
\[ \text{Primer 3: GCC CAA GGT ACC ACC GCC GTC AAC GTC GAG ACG TCA GGT GAG GGT GTC GCA GAT TTG CTR ACC CAR TCT CCA} \]

\[ \text{SacI} \]

\[ \text{Primer 4: GGC ACC ACG AGT GGT ATG GCA AAG AGG AGT TTT TTC ACT GAC TGT CCT TCC GTG ACA ACA TAT GGC GCG} \]
\[ \text{Primer 5: AGG TSM TGC GAC AGT CWG G} \]
\[ \text{Primer 6: TGA GGA GAC GGT GAC GTG CCC TTG GCC CC} \]
\[ \text{Primer 7: CTT TGC CAT ACC ACT GCT GGT GCC ATT TTA CTC CTC GCC GCG TGC CCA ACC AGG GAT GGC CAA GGT SMA RCT} \]
\[ \text{GCA GSA GTC WGG} \]
\[ \text{Primer 8: CAC AAT GTC GCT CCA GCC TCC ACC TGA GGA GAC AGT GAC TGT GGC ATT ACC TTG GCC} \]
\[ \text{XhoI} \]
\[ \text{Primer 9: CAT GCC ATG ACT GCG CCA GCC GCA GAT CAC GCA GGT TCA GCT GGT GAA GTC TGG} \]
\[ \text{SfiI} \]
\[ \text{Primer 10: GGT AGC TAG CTC ATG AGC TCA CGG TCA GAA GGG TTC CTT GAC C} \]
\[ \text{SacI} \]
\[ \text{Primer 11: GGT GGA GCC TCG AGC GAT ATC CAG ATG ACC CAG TCC CGG} \]
\[ \text{XhoI} \]
\[ \text{Primer 12: GAG TCA TTC TGC GCC CGC CCG TTT GAT TTC CAC CTT GTC GCC} \]
\[ \text{NotI} \]

All 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.

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-CD3α VH domain was first connected to the N-terminus of the anti-EGP2 VL domain using a 5' linker

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\[ \text{SacI} \]

\[ \text{Primer 4: GGC ACC ACG AGT GGT ATG GCA AAG AGG AGT TTT TTC ACT GAC TGT CCT TCC GTG ACA ACA TAT GGC GCG} \]
\[ \text{Primer 5: AGG TSM TGC GAC AGT CWG G} \]
\[ \text{Primer 6: TGA GGA GAC GGT GAC GTG CCC TTG GCC CC} \]
\[ \text{Primer 7: CTT TGC CAT ACC ACT GCT GGT GCC ATT TTA CTC CTC GCC GCG TGC CCA ACC AGG GAT GGC CAA GGT SMA RCT} \]
\[ \text{GCA GSA GTC WGG} \]
\[ \text{Primer 8: CAC AAT GTC GCT CCA GCC TCC ACC TGA GGA GAC AGT GAC TGT GGC ATT ACC TTG GCC} \]
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\[ \text{Primer 9: CAT GCC ATG ACT GCG CCA GCC GCA GAT CAC GCA GGT TCA GCT GGT GAA GTC TGG} \]
\[ \text{SfiI} \]
\[ \text{Primer 10: GGT AGC TAG CTC ATG AGC TCA CGG TCA GAA GGG TTC CTT GAC C} \]
\[ \text{SacI} \]
\[ \text{Primer 11: GGT GGA GCC TCG AGC GAT ATC CAG ATG ACC CAG TCC CGG} \]
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\[ \text{NotI} \]

All 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.
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 Mr 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 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 ± 0.04 × 10⁻² sec⁻¹, which was approx. 2-fold faster than that of the parental anti-EGP2 scFv (3.4 × 10⁻² 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” immuno-chemistry. 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)
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).

**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

![Diagram of "capture" immunohistochemistry](image)

**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 CD3ε 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).

![Immunohistochemical analysis of bispecific diabody binding](image)

**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 µm).

![In vitro cytolytic activity of activated T lymphocytes against EGP2+ GLC1M13 target cells](image)

**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.

![Lysis of GLC1M13 lung carcinoma cells mediated by anti-EGP2/anti-CD3 diabody Dia5v9 (black bars) or BsMAb F(ab')2 Bis1 (white bars) at different E:T ratios and Ab concentrations](image)

**Figure 7** – Lysis of GLC1M13 lung carcinoma cells mediated by anti-EGP2/anti-CD3 diabody Dia5v9 (black bars) or BsMAb F(ab')2 Bis1 (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 (± SD) of triplicate measurements.
immunotherapeutical concept has been studied extensively in pre-clinical model systems and in phase I clinical trials (reviewed in Boluis 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 an opposite end 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 carcinoma therapy, we have aimed to construct a small and partially humanized recombinant molecule with dual specificity for both an antigen EGP2 (alias CO17-1A antigen), and the signal transducing CD3 chain of the 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 of a panel of anti-EGP2 antibodies. This analysis showed that the hybodoma. 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 x 10^{-2} 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 EG2 showed an off-rate of 7.27 ± 0.04 x 10^{-4} sec^{-1}, which was approximately 2-fold faster than that of the parental scFv MOC31 (3.4 x 10^{-4} 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” immunohistochemistry 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 eEGP2 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 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 bi-specificity of the diabody binding the cytotoxicity of Dia5v9 is comparable with an analogous hybrid-hybridoma bispecific antibody Bi1 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 cytolyis 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 cytolyis, the distance (6.5 nm) between target and cytolytic 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 Dia5v9 with the CTLL2/humCD3 transfectant cell line (CTLL2/humCD3). 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).

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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 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 (McGuinnes 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 chain transfectant cell line CTL2/L2/humCD3e. A rough comparison of IC50 obtained using scFv-OKT3 (Kipriyanov et al., 1997) with that of Fab-UCHT1v9 (Zhu and Carter, 1995) shows an approximate, 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 Dia5v9 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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