Generation of chimeric bispecific G250/anti-CD3 monoclonal antibody, a tool to combat renal cell carcinoma

RM Luiten1, LR Coney2, GJ Fleuren1, SO Warnaar1 and SV Litvinov1

1Department of Pathology, University of Leiden, University Hospital Building 1, L1-Q, PO Box 9600, 2300 RC Leiden, The Netherlands; 2Apolinn Inc., Malvern, PA 19355, USA.

Correspondence: RM Luiten
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Summary The monoclonal antibody (MAb) G250 binds to a tumour-associated antigen, expressed in renal cell carcinoma (RCC), which has been demonstrated to be a suitable target for antibody-mediated immunotherapy. A bispecific antibody having both G250 and anti-CD3 specificity can cross-link G250 antigen-expressing RCC target cells with T cells and can mediate lysis of such targets. Therapy studies with murine antibodies are limited by immune responses to the antibodies injected (HAMA response), which can be decreased by using chimeric antibodies. We generated a chimeric bispecific G250/anti CD3 MAb by transfecting chimeric genes of heavy and light chains for both the G250 MAb and the anti-CD3 MAb into a myeloma cell line. Cytotoxicity assays revealed that the chimeric bispecific MAb was capable of mediating lysis of RCC cell lines by cloned human CD8+ T cells or by IL-2-stimulated peripheral blood lymphocytes (PBLs). Lysis mediated by the MAb was specific for target cells that expressed the G250 antigen and was effective at concentrations as low as 0.01 μg ml⁻¹. The chimeric bispecific G250/anti-CD3 MAB produced may be an effective adjuvant to the currently used IL-2-based therapy of advanced renal cell carcinoma.

Keywords: chimeric bispecific monoclonal antibody; renal cell carcinoma; immunotherapy; G250 antigen

Targeting cytotoxic effector cells for localisation in tumours has been pursued as an attractive therapeutic option. One approach is the use of bispecific monoclonal antibodies that can cross-link tumour cells with activation-related molecules on effector cells, such as the CD3 complex on cytotoxic T lymphocytes (CTLs) or with Fc receptors on monocytes and natural killer (NK) cells (Fanger et al., 1990). Cross-linking of CD3 by bispecific antibodies can redirect the specificity of activated T lymphocytes and circumvent MHC restriction (Garvido et al., 1990; Berg et al., 1991; Brissiencek et al., 1991; Qian et al., 1991; Nistico et al., 1992). It has been shown that intravenously administered bispecific MoV18/anti-CD3 (OC/ TR) MAb, recognising both the ovarian carcinoma-associated antigen MoV18 and the CD3 complex, can localise in tumours in patients (Tibben et al., 1993). Targeted T lymphocytes, bearing bispecific antibody bound to their CD3 complex, may accumulate in the tumour, whereas any bispecific MAb binding to tumour cells first (Van Dijk et al., 1991), may subsequently attract circulating T cells.

Monoclonal antibody (MAb) G250 recognises an antigen expressed on human renal cell carcinoma (RCC, Oosterwijk et al., 1986). Whole immunoglobulin molecules and F(ab)2 fragments of the G250 MAb localised preferentially in tumours, both in model studies with RCC xenografted nude mice and RCC patients, and were suitable for tumour visualisation in mice (Van Dijk et al., 1988, 1991) and in patients (Oosterwijk et al., 1993). Therapeutic effects of murine G250 IgG2a were demonstrated by tumour growth inhibition studies in nude mice xenografted with RCC (Van Dijk et al., 1994). The G250 antigen can therefore be considered a suitable target for antibody-mediated immunotherapy. Murine bispecific G250/anti-CD3 antibody has previously been generated and was shown to induce lysis of RCC cell lines by IL-2-activated human CTLs in vitro (Van Dijk et al., 1989). In nude mice F(ab)2 fragments of the murine bispecific G250/anti-CD3 MAb localised well in xenografted RCC (Van Dijk et al., 1991).

To cure established RCC will require intensive treatment with multiple MAb injections over an extended period. Repeated administration of murine antibodies to patients frequently elicits a human anti-mouse antibody (HAMA) response (Courtney-Luck et al., 1986; Schroff et al., 1985; Shawler et al., 1985; Saleh et al., 1993; Riva et al., 1993). Chimeric antibodies, in which the murine constant regions of the heavy and light chain are replaced by those of human origin, are less immunogenic and have a longer serum half-life than murine antibodies (LoBuglio et al., 1989; Meredith et al., 1991). The strong immunogenicity of murine G250 and anti-CD3 MAbs observed in patients, was absent when the chimeric versions of these antibodies were injected (Oosterwijk et al., 1993; Canavari et al., 1995; E Oosterwijk, personal communication; Coney et al., 1996). Based on the observation that chimerisation abrogates the immunogenicity of the parental G250 and the anti-CD3 MAb in patients, we expect the chimeric bispecific G250/anti-CD3 MAb to be non-immunogenic.

We generated a chimeric version of the bispecific G250/anti-CD3 antibody to enable treatment of patients with the bispecific G250/anti-CD3 MAb. To this end the non-immuno-globulin-producing myeloma cell line, P3X-63Ag.653, was transfected with expression vectors encoding the chimeric heavy and light chains of the anti-CD3 and the G250 antibody. Transfectants that produced bispecific antibody were selected and were used for antibody purification. The chimeric bispecific antibody was found to bind specifically to the G250 antigen expressed on RCC cell lines and to CD3 on human T lymphocytes. In vitro cytotoxicity assays showed that the chimeric bispecific G250/anti-CD3 MAb is capable of inducing lysis of G250-positive RCC cell lines by IL-2-activated human T lymphocytes as well as peripheral blood lymphocytes (PBLs).

Materials and methods

Cell lines

The non-immunoglobulin-producing murine myeloma cell line P3X63Ag.653 (ATCC CRL 1580) and the T cell line HPB-ALL were cultured in Iscove's modified Dulbecco's medium supplemented with 2 mM l-glutamine and 5% heat-inactivated fetal calf serum (FCS). The human RCC cell lines A704 (ATCC HTB45), ACHN (ATCC CRL1611), SK-RC-1, SK-RC-7 and SK-RC-45, obtained from the Sloan Kettering...
Memorial Institute (NY, USA), were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS and penicillin/streptomycin (P/S, GIBCO-BRL Life Technologies, Breda, The Netherlands). Adherent cells were passaged at confluence with 0.5% trypsin and 0.01% EDTA. The human CD3+CD8+ T cell clone, TIL 7.9, was derived from tumour-infiltrating lymphocytes of a cervical carcinoma (kindly provided by W van Driel, Leiden, The Netherlands). The TIL 7.9 cells were cultured in 96-well round bottom microtitre plates in RPMI-1640 medium containing 10% human AB serum, P/S, 4 mM L-glutamine, 1 μg ml⁻¹ leucaaglutinine, 1 μg ml⁻¹ indomethacin, 10 U ml⁻¹ IL-2 (Eurocetus, Amsterdam, The Netherlands) in the presence of feeder cells irradiated with 30 Gy. The feeder cell population contained the EBV-transformed human B cell lines, APD and BSM (kindly provided by Dr R Bolhuis, Rotterdam, The Netherlands), pooled human PBLS and the autologous cervical carcinoma cell line, CSCC-7, at concentrations of 20,000 cells per well for PBLS and 5000 cells per well for the B cell lines and tumour cells respectively. The transfectoma cell lines anti-CD3 4B5, producing chimeric anti-CD3 MAb, and chG250 7C1, producing chimeric G250 MAbs, were kindly provided by Centocor (Leiden, The Netherlands), and were cultured in RPMI mixed with DMEM in a 1:1 volume ratio, supplemented with 10% FCS, P/S, 4 mM L-glutamine and 0.02 mM β-mercaptoethanol.

**Expression vectors**

Expression vectors containing the chimeric heavy and light chain genes for the G250 MAbs, pSVgpgHGI-G250 (chimeric heavy chain vector) and pSVgpgHCkG250 (chimeric light chain vector), and the anti-CD3 MAb, pSVgpgHGI-CD3 (heavy chain) and pSVneoHCkCD3 (light chain), had previously been constructed (Coney et al., 1996). The chimeric genes were composed of the murine V₄ and V₅ regions connected to the human C₄ and C₅ regions respectively. The anti-CD3 coding genes had previously been derived from the OC/TR cell line, described by Mezzanzanica et al. (1988). The pSVneoHCkCD3 vector contained the neo gene for selection of G418 resistant transfectants. The other vectors contained the xanthine guanine phosphoribosyl transferase (gpt) gene, that confers resistance to mycosporphinic acid after transfection into mammalian cells. To construct a double plasmid, pSVgpgHGI-G250/HCKG250, containing the chimeric G250 IgG1 heavy as well as the light chain genes, the 10 kb SalI restriction fragment from pSVgpgHCKG250, containing the chimeric light chain gene construct, was isolated and inserted into the SalI site of pSVgpgHGI-G250. The presence of the insert and its orientation with respect to the chimeric heavy chain gene was checked by restriction analysis with SalI, Xhol/EcoRI and EcoRI/EcoRV restriction enzymes (Figure 1).

**Transfections**

An aliquot of 10 μg of pSVgpgHGI-CD3 and pSVneoHCkCD3 plasmid DNA was linearised by digestion with respectively KpnI and BamHI endonucleases, extracted with chloroform/phenol, ethanol precipitated and dissolved in sterile water at a final concentration of 1 μg ml⁻¹. pSVgpgHGI-CD3 was additionally digested with EcoRV to cleave out a 250 bp fragment, which inactivates the gpt gene. Resting myeloma P3X63Ag8.653 cells were seeded at a density of 2 x 10⁶ cells ml⁻¹ 24 h before transfection. Cells were washed twice in Hank's, balanced salt solution (BSS) and resuspended at 2 x 10⁶ cells ml⁻¹. An aliquot of 10 μl of pSVgpgHCkCD3 was mixed with 10 μl pSVneoHCkCD3 in a prechilled cuvette and 1 ml of cells was added. Cells were electroporated at 200 V and 960 μF in a BioRad Gene Pulser (BioRad, Hercules, NY, USA). The cell suspension was diluted with 9 ml of cold culture medium and incubated on ice for 30 min. Living cells were counted and plated out in flat bottom 96-well microtitre plates at a density of 10,000, 5000 and 2500 cells per well. Cells were cultured at 37°C and 5% carbon dioxide for 48 h, followed by the addition of G418 (0.5 mg ml⁻¹). G418-resistant transfectants were screened for chimeric antibody production by ELISA after 2 weeks and the transfectant that produced the highest levels of chimeric anti-CD3 MAb was retransfected with 10 μg Xhol-linearised pSVgpgHGI-G250/HCKG250 double plasmid. Transfectants were selected in medium containing 0.25 μg ml⁻¹ mycosporphinic acid, 1.25 μg ml⁻¹ hypoxanthine, 25 μg ml⁻¹ xanthine and 0.5 mg ml⁻¹ G418. Production of chimeric G250 MAbs was analysed by ELISA. The transfectant with the highest yield of chimeric G250 MAbs, as determined by ELISA, was cloned by limiting dilution and clones were assayed for chimeric G250 MAb production by ELISA. The production of chimeric bispecific MAb was analysed by flow cytometry and the functionality of the chimeric bispecific MAb was tested in a cytotoxicity assay. Stability of antibody production was determined after 2 months of culture by determining the percentage of bispecific MAb producing subclones after a limiting dilution cloning.

**ELISA**

For detection of chimeric anti-CD3 antibody in the medium of the transfectants, ELISA plates, coated at 4°C for 24 h with 1 μg per well goat anti-human IgG, Fc fragment-specific (Jackson ImmunoResearch Laboratories, Westgrove, PA, USA) in coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate pH 9.55), were washed with phosphate-buffered saline (PBS), 0.05% Tween and incubated with 50 μl of culture supernatant of the transfectants for 1 h at room temperature. Plates were washed and incubated with 50 μl per well alkaline phosphatase-conjugated goat anti-human...
IgG (H+L) as secondary antibody (1:1000, Jackson ImmunoResearch Laboratories). Binding of secondary antibody was detected by incubation at room temperature for 30 min with 1 µg ml⁻¹ p-NPP phosphatase substrate solution (100 µl per well, Sigma 104 substrate tablets in sterile saline supplemented with 0.2% alkaline buffer solution, Sigma, St Louis, MO, USA). The reaction was terminated by adding 50 µl per well 3 M sodium hydroxide and the optical density (OD) at 410 nm was recorded in an ELISA plate reader (Dynatech, Chatilley, VA, USA). Purified chimeric antibody of the IgG1 isotype was used for a standard curve to calculate the antibody concentration in the samples. Anti-idiotypic G250 MAb (clone NUH-9, obtained from Dr E Oosterwijk, 0.25 µg per well) was used in ELISA as coating antibody for the detection of chimeric G250 and bispecific antibody.

Reverse transcriptase–polymerase chain reaction (RT–PCR)

mRNA was isolated from 3 x 10⁷ cells using the Micro-Fast Track mRNA isolation kit (Invitrogen, Leek, The Netherlands) according to instructions supplied and was subsequently dissolved in 10 µl of sterile water. Per cell line 1 µl of mRNA was converted into cDNA by reverse transcription using the cDNA Cycle kit (Invitrogen) in 20 µl reaction volume. For PCR on the cDNA, primer sets were designed, specific for the variable regions of heavy and light chains of the anti-CD3 and G250 MAb respectively (Pharmacia Biotech, Roosendaal, The Netherlands). The human Cy primer, 5’-GGGGAGACCGATGGCCCTTG-3’, derived from the DNA sequence of the human constant region for IgG, is a universal primer for the human IgG heavy chain constant region. This primer was used as 3’ primer in combination with either the CD3VH primer, 5’-CAGGTCGCACTCTGCAGTC-3’ or the G250VH primer, 5’-GGGGAGGCTTAGTGAAGCT-3’ as 5’ primer for specific amplification of, respectively, the anti-CD3 or G250 antibody heavy chain. PCR reactions with these primers were performed in a buffer containing 60 mM TrisHCl, 15 mM ammonium sulphate and 1.5 mM magnesium chloride (pH 8.5) at an annealing temperature of 58°C. An aliquot of 2 µl of the reverse transcriptase reaction mixture was used per

Figure 2  Flow cytometric analysis of chimeric bispecific MAb in culture supernatant of transfectants. HPB-ALL T cells were incubated with culture supernatant of transfection T3 no. 36.4 (white) or PBS (black) followed by incubation with either anti-idiotypic G250 MAb and FITC-labelled goat anti-murine IgG1 MAb (a) or FITC-labelled goat anti-human IgG MAb (b). c, Control incubations of HPB-ALL T cells with chimeric G250 (black), chimeric anti-CD3 MAb (grey) or bispecific G250/OKT-3 F(ab)₂ fragments (white), followed by an incubation with anti-idiotypic G250 MAb and FITC-labelled goat anti-murine IgG1 MAb. d, Control incubations with chimeric G250 (black), chimeric anti-CD3 MAb (white) or bispecific G250/OKT-3 F(ab)₂ fragments (grey), followed by an incubation with FITC-labelled goat anti-human IgG MAb.
PCR reaction. For kappa light chain amplifications the human cκ5 primer, 5'-GATGGTGACTCCTGGAGGGG-3', derived from the DNA sequence of the human kappa chain constant region, was used in combination with either the CD3κ 3' primer, 5'-GTCGTGCATCTCCAGGGG-3' or the G250κ 3' primer, 5'-CAGTGGGACAGGGT-3' at an annealing temperature of 55°C. The anti-CD3 kappa chain amplification was amplified in the buffer described for the heavy chain PCR; the G250 kappa chain PCRs were performed in a buffer containing 60 mM Tris-HCl, 15 mM ammonium sulphate, 2 mM magnesium chloride, pH 9.5. All primers were used at a final concentration of 50 ng 25 μl⁻¹ in 25 μl PCR reaction volume. Thirty cycles of 1 min melting at 94°C, 1.5 min annealing and 2.5 min elongation at 72°C, were performed per reaction. PCR products were visualised by performing 2% agarose gel electrophoresis and staining the gel with ethidium bromide.

**Flow cytometry**

The presence of chimeric bispecific antibody in the culture supernatant of the transfectants was analysed by flow cytometry. Aliquots of 5 x 10⁶ HPB-ALL T cells were incubated with 50 μl spent culture supernatant for 4 h. Concentrated spent culture supernatant of a chimeric G250-producing transfectant, purified chimeric anti-CD3 MAb, murine G250 MAb and murine bispecific G250/OCT-3 F(ab); fragments (van Dijk et al., 1989) were used as controls at concentrations of 20 μg ml⁻¹ IgG in a final volume of 100 μl. After two washes cells were incubated with either 40 μg ml⁻¹ anti-idiotype G250 MAb (NUH-9) or 20 μg ml⁻¹ goat anti-human IgG, FITC-labelled, for 1 h. Bound anti-idiotype G250 MAb was stained with FITC-labelate, goat anti-murine IgG1, heavy chain specific. Incubations with FITC-labelled antibodies were performed in the dark. Cells were washed twice, resuspended in 500 μl PBS and analysed on a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). RCC cell lines were stained for G250 antigen expression using murine G250 IgG1, which was subsequently detected with goat anti- murine IgG labelled with FITC. Dead cells were stained with propidium iodide and were excluded from analysis.

**Antibody purification**

T3 no. 36,4 cells, producing chimeric bispecific G250/anti-CD3 MAb, were cultured in a 1 – 1.5 l culture volume and the supernatant was concentrated ten times using a ProVari-o-3 filter system with a 70 kDa membrane cassette (Filtron Technology, Terheiden, The Netherlands). Chimeric IgG1 was purified from the concentrated supernatant on a protein A - Sepharose column using 0.1 M citrate buffer at pH 3.0 for antibody elution. The bispecific antibody fraction was separated from parental antibody by high pressure liquid chromatography (HPLC) on Mono S - Sepharose using a sodium chloride gradient from 0 to 0.2 M in 25 mM sodium acetate, pH 5.0, a matrix and procedure that had previously been described (van Ravenswaay Claesen et al., 1993; Warnaar et al., 1994; van Dijk et al., 1989).

**Cytotoxicity assays**

Approximately 2 x 10⁵ target cells were labelled with 50 μCi of ¹ⁱ⁵I (¹ⁱ⁵Iodide) at a concentration of 10⁴ cpm ml⁻¹ in 200 μl RPMI-1640 medium. The cells were washed twice and were resuspended in culture medium at 2 x 10⁴ cells ml⁻¹. The human CD3⁺CD8⁺ T cell clone, TIL 7.9, was used as effector cells after 7 days of culture. Alternatively, human peripheral blood lymphocytes (PBLs) from healthy donors precultivated by culturing in RPMI-1640 with 10% human AB serum and 100 U ml⁻¹ IL-2 for 6 days, were taken as effector cell population. Aliquots of 2000 target cells were mixed with effector cells at effector : target ratios ranging from 100:1 to 12:1 in 96-well round bottom microtitre plates (Greiner, Langenthal, Switzerland). Serial 10-fold dilutions of antibody were added in concentrations ranging from 1 μg ml⁻¹ to 1 ng ml⁻¹. The plates were then centrifuged at 800 r.p.m. for 2 min and incubated at 37°C for 4 h. An aliquot of 100 μl supernatant was counted at 15 min in a LKB gamma counter (ER, experimental release). Spontaneous release (SR) and maximal release (MR) were assayed by respectively adding medium or 100 μl of 10% Triton X-100 to the labelled targets. The percentage specific lysis was calculated as (ER - SR/ MR - SR) x 100%.

**Results**

**Generation of chimeric bispecific G250/anti-CD3 MAb**

Chimeric heavy and light chain genes of anti-CD3 MAb were transfected into P3X63Ag8.653 myeloma cells, yielding transfectants producing chimeric anti-CD3 MAb, as was determined by ELISA. The transfectant that had produced the highest amount of antibody (25 μg per 10⁶ cells) during a 10 day culture, was selected and retransfected with the expression vector containing the chimeric heavy and light chain genes of the G250 MAb (Figure 1). The presence of antibodies with G250 specificity in the culture supernatant of the transfectants was analysed by ELISA using plates coated with anti-idiotype G250 MAb and goat anti-human IgG MAb as detecting antibody as described in Materials and methods. Twenty transfectants that produced antibodies with G250 specificity were selected for analysis of bispecific G250/ anti-CD3 MAb production.

The production of chimeric bispecific antibody was analysed by flow cytometry using HPB-ALL T cells, which express the CD3 antigen. HPB-ALL T cells were incubated with culture supernatant of the transfectants followed by the incubation with anti-idiotype G250 MAb (Figure 2a). Control incubations of HPB-ALL T cells with only anti-idiotype G250 MAb revealed that the anti-idiotype G250 MAb did not react with the HPB-ALL cells, and therefore...
was suitable to detect bispecific antibodies bound to the HPB-ALL T cells. This was confirmed by positive control
stainings with murine bispecific G250/OKT-3 (anti-CD3) MAb (Figure 2c). Chimeric G250 MAb did not react with
HPB-ALL T cells, as analysed with the anti-idiotype G250 MAb. A low level of cross-reactivity of the anti-idiotype
G250 MAb with the anti-CD3 MAb was observed (Figure 2c). Culture supernatant of several transfectants showed an
increase in fluorescence as compared with the control incubations of HPB-ALL T cells without culture super-
natant, indicating that these transfectants produced anti-

bodies with both G250 and anti-CD3 specificity (Figure 2a). In parallel, HPB-ALL cells, incubated with culture super-
natant or control antibodies, were stained with goat anti-

human IgG MAb as detecting antibody to determine the
total amount of antibodies with anti-CD3 specificity present
in the culture supernatant of the transfectants (Figure 2b and
d).

Subsequently, the capacity of culture supernatant of the
transfectants producing chimeric bispecific cG250/anti-CD3
MAb to trigger activated human CD8+ T cells for lysis of
target cells that express the G250 antigen was analysed in a
cytotoxicity assay. Figure 3 shows the results of supernatant
of transfectant T3 no. 36.4. The chimeric bispecific G250/
anti-CD3 MAb greatly increased the lysis of A704 target by
activated T cells, as was observed for the control murine
bispecific G250/OKT-3 (anti-CD3) MAb at effector–target
ratios ranging from 50:1 to 6:1.
Cytotoxicity of cloned human T cells mediated by chimeric bispecific MAb

Both antibody fractions (peaks 1 and 2) of the HPLC separation of the chimeric bispecific MAb were then analysed for their ability to mediate cytotoxicity by human T cells against G250 antigen-positive target cells. The antibody fraction of peak 2 was capable of mediating lysis of A704 target cells, whereas peak 1 antibody was not (Figure 5). Induction of lysis mediated by peak 2 (cG250/anti-CD3) was still detectable at an antibody concentration of 0.01 µg ml$^{-1}$ (Figure 5), which shows that bispecific antibody-mediated cytotoxicity is effective at low doses of antibody.

Tumour cell lines that did not express the G250 antigen were not lysed in the presence of the bispecific antibody. The chimeric bispecific MAb mediated lysis of target cells with a relatively low expression of the G250 antigen as well as targets with a higher expression of the G250 antigen (Figure 6). The efficiency of lysis of G250-expressing cells, induced by chimeric bispecific MAb, was dependent on the concentration of the bispecific MAb as well as on the target cell line used.

As low levels of G250 expression on T cells might lead to cytokine release and autokill of T cells by the chimeric bispecific G250/anti-CD3 MAb, we have tested TNF-α release by T cells in the presence of the bispecific MAb, using the WEHI assay (Luiten et al., 1996). No TNF-α release was seen at concentrations of 0.1 µg ml$^{-1}$ bispecific MAb or parental G250 MAb, whereas chimeric anti-CD3 MAb did lead to TNF-α release.

Based on the binding specificities of both antibody peaks and the difference in capacity to induce cytotoxicity by T cells, peak 2 was considered to contain chimeric bispecific G250/anti-CD3 MAb.

Analysis of mRNA expression for antibody heavy and light chains

In the chromatogram of the HPLC separation of the chimeric bispecific antibody fractions a third peak, representing the parental anti-CD3 MAb, was absent. This might indicate that one of the chains for the anti-CD3 MAb was not produced by clone T3 no. 36.4, inhibiting the production of parental anti-CD3. Therefore, the presence of anti-CD3 and G250 MAb heavy and light chain mRNA in the chimeric bispecific MAb-producing transfectant, T3 no. 36.4, was determined by RT-PCR using primer sets specific for the variable regions of the G250 or the anti-CD3 MAb heavy and light chains.

The parental cell line and cell lines producing either chimeric anti-CD3 MAb or chimeric G250 MAb were used as control cell lines to check the specificity of the primers. Figure 7 shows that the primer sets specific for the variable regions of anti-CD3 MAb heavy and light chains generated a PCR product of mRNA derived from the anti-CD3 MAb-producing cell line as well as of mRNA derived from the chimeric bispecific-producing clone, T3 no. 36.4, indicating that the transfectant contains mRNA for both anti-CD3 antibody chains.

In the control experiment these primer sets did not yield a PCR product with the G250 MAb-producing cell line not containing anti-CD3 MAb mRNA. Analogous results were obtained in analyses for the G250 antibody mRNA. From both the G250 MAb-producing cell line and the clone T3 no. 36.4 a PCR product was obtained with primer sets specific for either the heavy or light chain variable regions of G250 MAb, and not from the anti-CD3 MAb-producing cell line. Clone T3 no. 36.4 appeared to contain mRNA for heavy and light chains of both the anti-CD3 and the G250 MAb, indicating that all chains transfected were expressed in clone T3 no. 36.4.

Cytotoxicity of human PBLS mediated by the chimeric bispecific MAb

Bispecific antibody-mediated cytotoxicity in vivo will deal with lymphocytes of various phenotypes as effector cell population.
Figure 6 Lysis of RCC cell lines by cloned human T cells in the presence of chimeric bispecific MAb. The RCC cell lines, A704, SK-RC-1, SK-RC-7, SK-RC-45 and ACHN were stained for G250 expression and analysed by flow cytometry (a and b). TIL7.9 cells were assayed for cytotoxicity against A704 (○), SK-RC-1 (●), SK-RC-7 (+), SK-RC-45 (△) and ACHN (▲) in the presence of 1, 0.1, 0.01, 0.001 or 0 μg ml⁻¹ chimeric bispecific G250/anti-CD3 MAb. E/T ratio 25:1, 2000 targets per well (c). d, G250 antigen expression of RCC cell lines. Mean channel number (MCN) after subtraction of autofluorescence.

To show that our chimeric bispecific antibody is capable of inducing lysis by a heterogeneous population of lymphocytes, peripheral blood lymphocytes (PBLs) of different donors were used as effector cells. Since chimeric bispecific MAb was unable to induce cytotoxicity with unstimulated PBLs or with PBLs after an overnight stimulation with 100 U ml⁻¹ IL-2 (van Ravenswaay Claassen et al., 1993), PBLs were cultured with 100 U ml⁻¹ IL-2 for 6 days before cytotoxicity assays. Figure 8 shows that the chimeric bispecific G250/anti-CD3 MAb induced significant lysis of A704 targets with PBLs from donor A or donor C compared with controls without antibody, whereas PBLs from donor B showed a marginal enhancement of lysis. Parental chimeric anti-CD3 MAb and G250 MAb do not mediate cytotoxicity of activated PBLs at the concentration of 1 μg ml⁻¹ (data not shown). Activation of PBLs from donor A, but not donor B, enhanced the level of lysis of SK-RC-7 target cells mediated by chimeric bispecific G250/anti-CD3 MAb (data not shown). The amount of lytic activity that was induced in a 4 h incubation varied between donors (van Ravenswaay Claassen et al., 1993). This might be owing to differences in the percentage of CD8⁺ cells among the IL-2-stimulated PBLs. Flow cytometric analysis revealed that PBLs from donor A indeed contained more CD8⁺ T cells (45%) than PBLs from donor B (24%). These results show that IL-2-activated PBLs can be triggered to lyse G250 antigen-positive RCC target cells in the presence of bispecific cG250/anti-CD3 MAb.

Discussion

Renal cell carcinoma has been studied intensively to evaluate different approaches to immunotherapy. Phase I and II clinical trials in which patients with RCC were treated with IL-2 revealed the susceptibility of RCC to IL-2, but also showed the toxicity of this cytokine when administered systemically in high doses. IL-2-based therapy is therefore limited by maximum tolerated dose (Gaynor et al., 1990). The response rates between these trials varied between 12 and 30% (Marumo et al., 1989; Hayat et al., 1991; Jensen et al., 1990; Whitehead et al., 1990; Rosenberg et al., 1989), but occasionally no responses were observed (Abrams et al., 1990). Co-administration of lymphokine-activated killer (LAK) cells (Parkinson et al., 1990; Fisher et al., 1988; Foon et al., 1992; Palmer et al., 1992) or tumour-infiltrating lymphocytes (TILs) from RCC (Bukowski et al., 1991) did not result in a significantly better response rate. Several other cytokines, such as interferon (IFN)-α (Muss et al., 1987; Feruglio et al., 1992), IFN-β and IFN-γ (Ernstoff et al., 1992), have been studied for anti-tumour effects in RCC patients. Clinical data of IFN-β treatment combined with IL-2 suggest a better response rate than obtained with IFN-β or IL-2 therapy alone (Kriegl et al., 1990). These clinical data show that RCCs are responsive to immunotherapy, but that the anti-tumour effects of cytokines are limited by the toxicity of the therapy used.

Directing effector cells towards the tumour by using bispecific antibodies is a method to increase the specificity of the effector cell population for the targeted tumour cells. Such bispecific MAbs have been demonstrated to induce effective lysis of tumour cells in vitro. Limited patient studies showed localisation of bispecific antibody-targeted effector cells to the tumour and cross-linking of effector cells with tumour cells (Kroesen et al., 1993). A study of intraperitoneal treatment of ovarian carcinoma with the bispecific OC/TR MAb which recognises both the carcinoma-associated antigen
complex regression murine tumour MOv18 development patients, al., bispecific G250/anti-CD3 (LoBuglio line demonstrated regression. MAb as CD3 picturesshow electrophoresed the low 1994). Chimeric bispecific MAb-targeted MAb generated ofthe G250 human celllinethat can encoding ofantibody can administered over a long treatment period hopefully resulting in more extensive tumour regression.

We generated a cell line that produces chimeric bispecific G250/anti-CD3 MAb stably by supertransfection of a cell line producing chimeric anti-CD3 MAb with a single expression vector encoding both chimeric heavy and light chains of the G250 MAb. The chimeric bispecific G250/anti-CD3 MAb produced by the supertransfected cells, was demonstrated to bind both to G250-expressing RCC cells and to human T cells. Chimeric bispecific G250/anti-CD3 MAb is capable of mediating lysis of G250 antigen-positive RCC cells by cloned human CD8+ T cells at concentrations as low as 0.01 μg ml⁻¹. Tumour cells with a relatively low expression of the G250 antigen were shown to be lysed equally as well as tumour cells with a high G250 antigen expression.

The chromatogram of the HPLC analysis of the antibody pool produced by clone T3 no. 36.4 revealed only two sharp and symmetrical peaks. One peak (1) corresponded to the chromatogram of G250 MAb and the other peak (2) represented the bispecific antibody fraction. The absence of a third peak representing parental anti-CD3 MAb was not the result of the loss of either the anti-CD3 MAb heavy or light chain, since RT-PCR analysis showed the presence of mRNA for all four antibody chains. The chromatographic separation method has been used previously to purify bispecific OTVL3/OKT-3 MAb (van Ravenswaay Claesen et al., 1993), murine bispecific OC/TR (Warnaar et al., 1994), chimeric bispecific OC/TR (Coney et al., 1996) and murine bispecific G250/OKT-3 MAb (van Dijk et al., 1994), and was shown to separate all combinations of heavy and light chains into distinguishable peaks. Therefore, we expect peak 2 of the chimeric G250/anti-CD3 MAb separation to contain IgG molecules composed of the parental heavy and light chains. We have no data to exclude mismatches in peak 2. However, De Lau et al. (1991) have shown that the production of two types of heavy and light chains by a quadrina does not necessarily lead to a random association of these chains. Depending on the combination of parental antibodies, preferential association of heavy and light chains may occur, resulting in the absence of some heavy and light chain combinations. In addition, the profiles of the chromatograms of different clones producing the same bispecific OC/TR MAb have been shown to differ greatly, as a result of different rates of synthesis of the antibody chains (Warnaar et al., 1994). In both the murine OC/TR-producing hybridoma and the chimeric OC/TR-producing hybridoma, clones were found that produced only one parental antibody and the bispecific MAb. The chromatogram of clone T3 no. 36.4 therefore represents one of the variants of the chromatograms that were observed among different clones producing other bispecific MAb.

For any RCC patient to be treated with chimeric bispecific
G250/anti-CD3 MAb, the effector cell population to be activated will be a T cell of variable phenotype residing in the PBLs. IL-2-activated PBLs can be activated in vitro by chimeric bispecific G250/anti-CD3 MAb to lyse G250 antigen-expressing RCC cells. The activated PBL cytotoxicity may vary between donors and may vary according to the activation protocol (van Ravenswaay Claasen et al., 1993). The in vitro cytotoxicity data suggest that treatment of RCC patients with chimeric bispecific G250/anti-CD3 MAb requires preactivation of the patients PBLs. Activated PBLs can then be administered to the patient in combination with chimeric bispecific G250/anti-CD3 MAb, or alternatively IL-2 can be given as in current IL-2-based regimens and bispecific G250/anti-CD3 MAb can be co-administered to enhance the effectiveness of IL-2-based therapy.

The murine versions of G250 and anti-CD3 MAb were immunogenic in patients, which was not the case for the chimeric versions (Dr E Oosterwijk, personal communication, Coney et al., 1996; Oosterwijk et al., 1993; Canevari et al., 1995). Since the components of the chimeric bispecific G250/anti-CD3 MAb did not induce an immune response, it is reasonable to expect that the chimeric bispecific MAb will also be non-immunogenic. Although humanisation of antibodies through CDR grafting is generally assumed to reduce immunogenicity further compared with chimeric antibodies, there is no solid patient data to support this notion. Humanised and chimeric antibodies have reduced immunogenicity compared with murine antibodies, however no direct comparison between humanised and chimeric MAbs has been performed. The fact that chimerisation of the G250 and anti-CD3 MAb apparently abrogates the patients’ immune response to the infused antibody, makes humanisation of the antibodies unnecessary. It is likely that any residual immunogenicity of chimeric or humanised antibodies will be idotype dependent.

In conclusion, the chimeric bispecific G250/anti-CD3 MAb may represent a useful tool for enhancing the results obtained in RCC treatment using IL-2.

Abbreviations
MAb, monoclonal antibody; RCC, renal cell carcinoma; HAMA, human anti-mouse antibody; Vh, variable region of antibody heavy chain; Vk, variable region of antibody kappa chain; C\textsubscript{H}, constant region of antibody heavy chain; C\textsubscript{K}, constant region of antibody kappa chain; gpt, xanthine guanine phosphoribosyl transferase; neo, neomycin; RT–PCR, reverse transcriptase–polymerase chain reaction.

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