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Induction of Tumour Cell Lysis by a Bispecific Antibody Recognising Epidermal Growth Factor Receptor (EGFR) and CD3

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A bispecific antibody construct (bAb) recognising CD3 and epidermal growth factor receptor (EGFR) was studied *in vitro*. Human peripheral blood lymphocytes (PBL), pre-activated with monoclonal antibody OKT-3 or with irradiated tumour cells, were armed with the bAb construct and targeted to autologous and allogeneic tumour target cells in culture. bAb EGFR×CD3 promoted significant cytolysis even at a concentration of 1 ng/ml. The specificity of target cell lysis was provided by the EGFR specificity of the bAb, as tumour cells negative for EGFR were not lysed. However, not only EGFR-positive tumour cells but also EGFR-positive normal cells were killed. Human renal cancer cell lines and the normal autologous kidney cell cultures expressing the same level of EGFR molecules were lysed to a similar extent. These results may contribute toward the planning of future clinical trials with such bAb.

Key words: bispecific antibodies, epidermal growth factor receptor, cytotoxic T lymphocytes
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INTRODUCTION

T LYMPHOCYTES ARE known as potent effectors in the immune response to cancer cells [1]. However, the role of cytotoxic T lymphocytes (CTL) in cancer patients is not well defined and remains a target for further research. Bispecific antibodies (bAb) have potential for tumour therapy since they can activate the lytic potential of a broad spectrum of CTL. In this regard, bAb recognising tumour-associated antigens and T cell markers were reported to bridge CTL and malignant cells *in vitro*, independent of CTL lytic potential or specificity [2, 3]. With the help of this

mechanism, CTL may be recruited to attack tumour cells. On T cells, the CD3 antigen is a prominent target molecule for T cell activation [2, 4–6]. On tumour cells, various tumour-associated antigens have been used as target molecules for bAb targeting. These include CA-19-9 for gastrointestinal carcinomas, ganglioside GD2 for malignant melanomas, folate-binding protein for ovarian cancers and N-CAM for gliomas and small cell lung cancers [7–10].

Increases in epidermal growth factor receptor (EGFR) gene expression and translation are observed in a variety of tumour cells. Thus, renal cell carcinoma, lung cancer, glioblastoma, breast cancer and squamous cell carcinoma express more EGFR than their corresponding normal tissues [11–15]. This accumulation of EGFR molecules was shown to correlate with unrestrained growth [13–15]. Several investigators also demonstrated that cell proliferation is dependent on endogenously produced transforming growth factor (TGF)-α which is a natural ligand for EGFR [16, 17]. In this study, we evaluated the potential of EGFR as a target structure for redirected cellular cytotoxicity by a bAb recognising EGFR and CD3.

MATERIALS AND METHODS

bAb

The murine IgG 2a monoclonal antibody (Mab) 425 recognises the human EGFR. The hybridoma was generated by Rodeck (The Wistar Institute, Philadelphia, U.S.A.) [18]. The anti-CD3 MAb hybridoma (OKT-3) was purchased from the ATCC (American Type Culture Collection, Rockville, Maryland, U.S.A.). bAb EGFRxCD3 was generated by chemical recombination of IgG fragments as described by Brennan *et al.* [19]. Briefly, anti-tumour and anti-T cell antibodies were converted into $F(ab')_2$ fragments by limited proteolysis with pepsin. $F(ab')_2$ fragments were purified by chromatography on protein A sepharose. Fab' fragments were generated by a mild reduction with dithiothreitol (DTT) (0.5 mM). The T cell-specific Fab' fragments were modified with 5,5'-dithio-bis-2-nitro-benzoic acid (DTNB). The $F(ab')_2$ bAb was generated by the conjugation of the Fab'–TNB (thio-bis-2-nitro-benzoic acid) derivative with the hinge-SH groups of the second Fab'-fragment. Purification of intermediate products (Fab', Fab'–TNB), as well as the final purification of bAb, was performed by gel filtration on a Superdex 75 column. Purity, homogeneity, as well as the bispecific composition of the products, were assessed by SDS–PAGE and chromatography on a hydroxylapatite column. The binding property to EGFR was tested using ELISA. Detection was performed after incubation with peroxidase-conjugated streptavidin and substrate. Reactivity to CD3 was investigated through fluorescence-activated cell sorter (FACS) analyses using Jurkat cells as CD3-positive targets.

Target cells

The human tumour cell lines MZ-MEL-6, MZ-MEL-11 (melanomas), MZ-CO-1 (colon cancer) and MZ-LC-1 (lung cancer) were established in our laboratory. The human normal kidney cell cultures MZ-NKC-1790 and MZ-NKC-1795, as well as the corresponding renal cancer cell lines MZ-RCC-1790 and MZ-RCC-1795, were also established by our group. The breast cancer cell line BT-20 and the erythroblastoid leukaemia cell line K-562 were purchased from the American Type Culture Collection. Culture conditions for human tumour and normal cells used in this study have been described previously [20].

Effector cells

Peripheral blood lymphocytes (PBL) were separated from heparinised blood of patients and healthy donors over a Ficoll density gradient by standard procedures. Stimulation of PBL with OKT-3 or MLTC was performed as recently described [8, 20].

Cytotoxicity assay

The cytotoxicity test used to analyse bAb was recently published [8]. Briefly, ^{51}Cr-labelled target cells were incubated with serially diluted bAb and effector lymphocytes. After incubation for 4 h at 37°C the percentage of specific ^{51}Cr release was calculated with the formula: % specific chromium release = (experimental ^{51}Cr release − spontaneous ^{51}Cr release) × 100/(maximum ^{51}Cr release − spontaneous ^{51}Cr release).

The role of natural killer (NK) cells for bAb-mediated cytotoxicity was determined by blocking experiments with unlabelled K-562 cells as competitors.

Flow cytometric analysis

FACS analyses of target cells with monoclonal antibody anti-EGFR were performed according to standard procedures.

RESULTS

bAb EGFR×CD3-induced cytotoxicity by autologous effector cells

Colon cancer cells were established as long-term culture (MZ-CO-1) from a surgical specimen of a patient. The cell line MZ-CO-1 shows high expression of EGFR. Autologous AF-PBL were stimulated *in vitro* with the autologous tumour cell line MZ-CO-1. Mixed lymphocyte tumour cell culture (MLTC)-

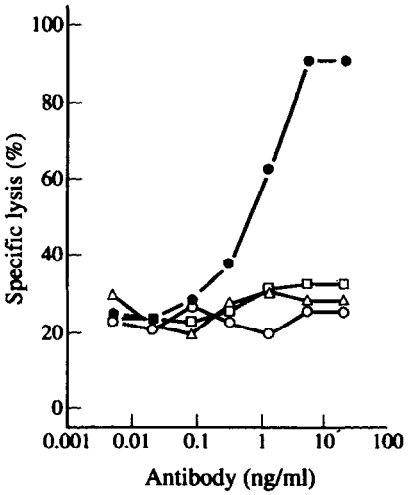

Figure 1. Cytolytic activity of autologous effector lymphocytes in the presence of bispecific antibody (bAb) EGFRxCD3. Effector cells: mixed lymphocyte tumour cell culture against autologous colon cancer cell line MZ-CO-1. Target cells: MZ-CO-1. E:T ratio = 90:1. Antibodies: bAb EGFRxCD3 (●), monoclonal antibody (MAb) α-EGFR (△), MAb α-CD3 (○), MAb α-EGFR plus MAb α-CD3 (□). Specific ^{51}Cr release of cytotoxic T lymphocytes without adding antibody (■).

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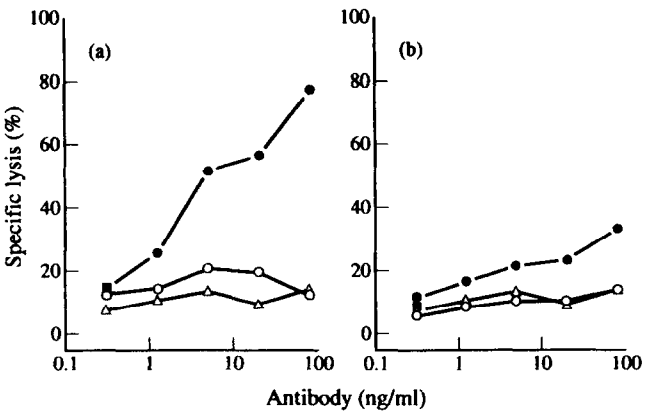

Figure 2. Cytolytic activity of various allogeneic effector populations in the presence of bispecific antibody (bAb) EGFRxCD3. Effector cells: mixed lymphocyte tumour cell culture against renal cell carcinoma MZ-RCC 1257 (a), OKT-3-stimulated peripheral blood lymphocytes from a patient suffering from renal cell carcinoma (b). Target cells: colon cancer cell line MZ-CO-1. E:T ratio = 30:1. Antibodies: bAb EGFRxCD3 (●), monoclonal antibody (MAb) α-EGFR (△), MAb α-CD3 (○). Specific ^{51}Cr release of cytotoxic T lymphocytes without adding antibody (■).

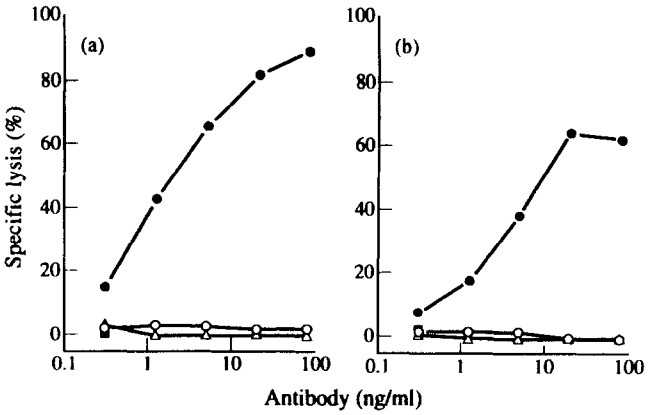

Figure 3. No influence of natural killer cells on cytolytic activity in the presence of bispecific antibody (bAb) EGFRxCD3. Effector cells: mixed lymphocyte tumour cell culture-activated lymphocytes against autologous colon cancer cell line MZ-CO-1. Target cells: MZ-CO-1 (a), MZ-RCC-1257 (b). E:T ratio = 90:1. K-562 blockade: K-562: target cell ratio = 80:1. Antibodies: bAb EGFRxCD3 (●), monoclonal antibody (MAb) α-EGFR (△), MAb α-CD3 (○). Specific ^{51}Cr release of cytotoxic T lymphocytes without adding antibody (■).

activated PBL lysed autologous cancer cells at a very low rate, with a maximum cytotoxicity of 25% specific ^{51}Cr release. The addition of bAb EGFRxCD3 to the MLTC-activated PBL enhanced the lysis of MZ-CO-1 up to 90% (Figure 1). Low concentrations of 1.0 ng/ml were sufficient to mediate cytotoxicity. Monoclonal antibodies $F(ab')_2$ and α-CD3, when used either as single agents or in combination, had no activity in the presence of effector cells.

bAb EGFR×CD3-induced cytotoxicity by allogeneic effector cells

Fresh PBL and PBL pre-activated *in vitro* by activation with MAb OKT-3 or MLTC were used as allogeneic effector cells. MLTC-activated PBL against autologous renal cell cancer MZ-RCC 1257, which did not demonstrate cross-reactive lysis to the colon cancer cell line MZ-CO-1, caused high cytolysis of MZ-CO-1 (80%) in the presence of bAb (Figure 2). Unstimulated PBL from various donors did not mediate lysis of EGFR-positive cancer cell line MZ-CO-1 in the presence of bAb EGFRxCD3. After *in vitro* stimulation of PBL with MAb OKT-3 alone for up to 11 days, tumour cell lysis of up to 35% was observed.

Influence of NK cell activity on bAb EGFR×CD3-induced cytotoxicity

In order to test the effect of NK cells on bAb-induced cytotoxicity, cold K-562 cells were used as unlabelled blocking targets for MLTC-activated PBL. bAb-induced cytolysis of the autologous target MZ-CO-1 and of the allgeneic target MZ-RCC-1257 was not affected by unlabelled K-562 cells (Figure 3). Therefore, the lytic potency of the tested effector cell populations in the presence of bAb was not due to NK-like activity.

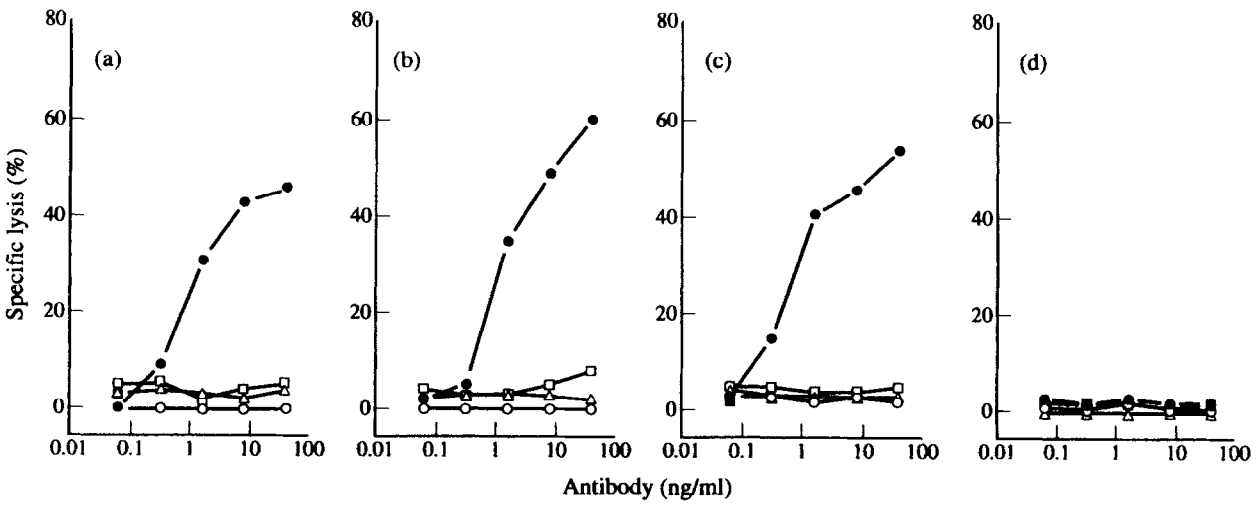

Figure 4. Cytolytic activity of *in vitro* activated lymphocytes against different tumour cells in the presence of bispecific antibody (bAb) EGFRxCD3. Effector cells: mixed lymphocyte tumour cell culture against autologous colon cancer cells MZ-CO-1. Target cells: EGFR ++ MZ-CO-1 (a), EGFR ++ MZ-LC-1 (b), EGFR + BT-20 (c), EGFR 0 MZ-MEL-6 (d). E:T ratio = 30:1. Antibodies: bAb EGFRxCD3 (●), monoclonal antibody (MAb) α-EGFR (△), MAb α-CD3 (○), MAb α-EGFR plus MAb α-CD3 (□). Specific ^{51}Cr release of cytotoxic T lymphocytes without adding antibody (■).

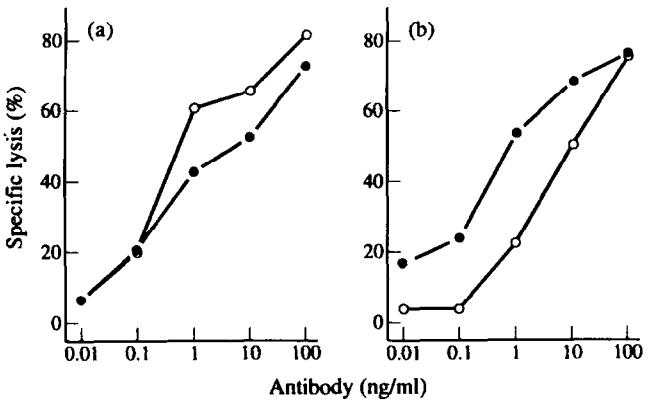

Figure 5. Cytolytic activity of *in vitro* activated lymphocytes against normal and malignant renal cells in the presence of bispecific antibody (bAb) EGFRxCD3 at different concentrations. Effector cells: mixed lymphocyte tumour cell culture against autologous colon cancer cells MZ-CO-1. Target cells: MZ-RCC 1790 (●) and MZ-NKC 1790 (○) (a); MZ-RCC 1795 (●) and MZ-NKC 1795 (○) (b). E:T ratio = 30:1. Antibody: bAb EGFRxCD3. Specific ^{51}Cr release of cytotoxic T lymphocytes without adding antibody: 12%.

Cytotoxicity of bAb EGFR×CD3 on tumour cells

The quantitative expression of EGFR in epithelial cancers varies. The tumour cell lines MZ-CO-1 (colon cancer), MZ-LC-1 (lung cancer), BT-20 (breast cancer) typed positive for EGFR, whereas the melanoma cell line MZ-MEL-6 was EGFR-negative. All tumour cell lines expressing EGFR were lysed by bAb-armed effector cells (Figure 4). There was no correlation between the amount of EGFR expression and the level of cytotoxicity. The EGFR-negative melanoma MZ-MEL-6 cell line was not lysed by bAb EGFRxCD3.

Cytotoxicity of bAb EGFR×CD3 on epithelial kidney cell cultures

Cytotoxicity assays with bAb-armed effector T cells on normal epithelial cells as targets were conducted. Both normal kidney cell cultures MZ-NKC-1790 and MZ-NKC-1795 and their corresponding autologous cancer cell lines MZ-RCC-1790 and MZ-RCC-1795 express EGFR. Specifically, FACS analyses revealed 42 and 69% EGFR expression on normal kidney cell lines MZ-NKC-1790 and MZ-NKC-1795, respectively, whereas their malignant counterparts MZ-RCC-1790 and MZ-RCC-1795 were 97 and 99% EGFR-positive. Cell lysis of malignant as well as normal kidney cell cultures was induced by bAb EGFRxCD3 in the presence of activated effector lymphocytes, as shown in Figure 5. Even when effector cell concentrations became limiting, selectivity for tumour cells was not revealed (Figure 6).

DISCUSSION

Previous studies have shown that tumour growth can be modulated by inhibiting EGFR-dependent cell proliferation. Thus, the regulatory pathway of renal carcinoma cell growth can be interrupted by a blocking antibody directed against EGFR [17]. Oncogenic poxviruses, coding for an EGF-like protein, can also be used as inhibitors that compete with natural ligands of EGFR [21]. In this regard, interferon-α downregulates the number of EGFR molecules on the cell surface and reduces the affinity of EGF for its receptor [22]. Both mechanisms result in a blockade of cell proliferation. EGFR-dependent cell proliferation may also be influenced by targeting T cells to the EGFR molecule. Thus, a genetically-engineered hetero-conjugate between EGF and anti-CD3 antibody was reported to mediate lysis of EGFR-bearing tumour cells by human cytotoxic T lymphocytes [23].

In this report we describe the cytolysis of EGFR-positive tumour cells mediated by a bAb, reactive with EGFR and CD3 antigen present on T lymphocytes. Tumour cell lysis can be induced by autologous as well as allogeneic effector cells. In this regard, OKT-3-stimulated PBL or MLTC-activated PBL are mediators of cell lysis in the presence of bAb. The enhanced cytotoxicity activity of MLTC-stimulated PBL compared to OKT-3-activated PBL may be due to CD8+ lymphocytes that are highly enriched by MLTC activation.

NK-like effector cells were found not to contribute to tumour cell lysis observed with bAb and activated T cells. Attempts to block NK cell activity *in vitro* with unlabelled K-562 cells as competitors had no influence on bAb-mediated tumour cell lysis. Similar results obtained after NK cell depletion have been described for other bAbs recognising the CD3 molecule [5]. Our

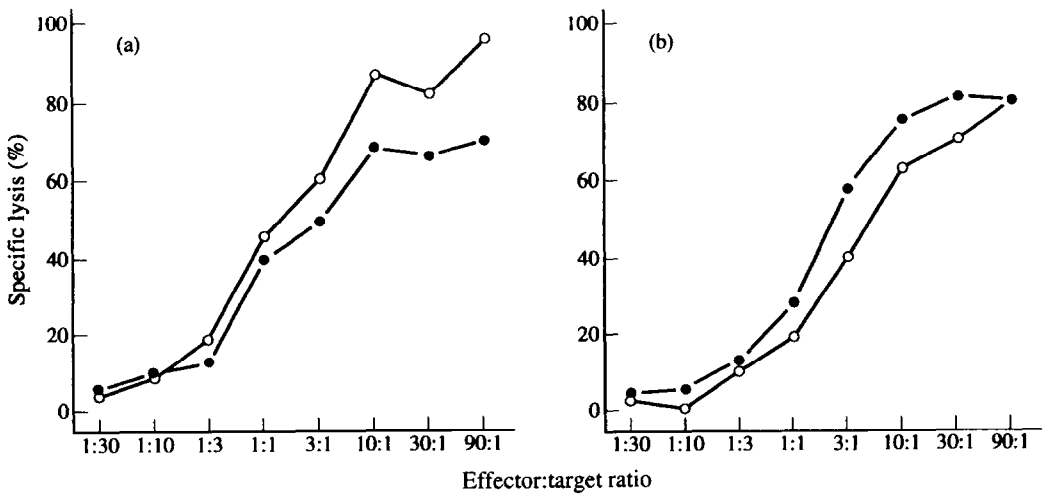

Figure 6. Redirected cellular cytotoxicity of *in vitro* activated lymphocytes against normal and malignant renal cells using different effector concentrations. Effector cells: mixed lymphocyte tumour cell culture against autologous colon cancer cells MZ-CO-1. Target cells: MZ-RCC 1790 (●) and MZ-NKC 1790 (○) (a); MZ-RCC 1795 (●) and MZ-NKC 1795 (○) (b). Antibody: bispecific antibody EGFRxCD3 40 ng/ml. Specific ^{51}Cr release of cytotoxic T lymphocytes without adding antibody: 11%.

results indicated that fresh and unstimulated PBL did not mediate tumour cell lysis, unless such cells were stimulated *in vitro*. Similar observations have been reported by Ferrini and coworkers [24]. The objective of future studies will be to recruit possibly all resting cytotoxic T cells by activation. Meanwhile, several groups have extended the repertoire of suitable effectors for targeting tumour cells by using additional activation markers such as CD2, CD28, CD16 or CD64 [25]. In order to evaluate the potential of the T cell antigen CD2 as a target structure for redirected cellular cytotoxicity, we have started to prepare bAb consisting of anti-CD2 and anti-EGFR antibodies.

In the experiments described here we found that tumour cell elimination induced by bAb depended on EGFR expression on target cells. Thus tumour cells which fail to express EGFR, such as melanotic melanoma cells, are not lysed by the EGFR-specific antibody. However, there is no correlation between the number of expressed receptor molecules and the level of lysis achieved. bAb EGFRxCD3 mediates cell lysis of various tumour cells which express different amounts of receptor molecules on the surface. An explanation for this observation may be that the high affinity of the bAb causes low concentrations of antibody and a high cytolytic potential of antibody-linked T cells. Consequently, normal renal cells are lysed, as well by bAb EGFRxCD3, although their EGFR expression is less than that of renal cancer cells. Locoregional application of bAb for intracavitary treatment may become a feasible approach for cancer treatment. In this regard, complete remissions of malignant ascites from ovarian cancer were reported after intraperitoneal injection of bAb-armed lymphocytes [26, 27]. A preliminary report on glioma treatment with bAb that recognises CD3 and a glioma-associated antigen was recently published [10]. Further preclinical and clinical studies seem warranted to establish those bAb constructs that are potent agents for future clinical application.

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