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The development and purification of a bispecific antibody for lymphokine-activated killer cell targeting against the rat colon carcinoma CC531

Peter J. K. Kuppen[1], Alexander M. M. Eggermont[2], Katinka M. Smits[1], Jaap D. H. van Eendenburg[1], Sylvia P. G. Lazeroms[1], Cornelis J. H. van de Velde[3], Gert Jan Fleuren[1]

[1] Department of Pathology, University of Leiden, Leiden, The Netherlands
[2] Department of Surgery, Rotterdam Cancer Institute, Rotterdam, The Netherlands
[3] Department of Surgery, University of Leiden, Leiden, The Netherlands

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Abstract. In vivo targeting of lymphokine-activated killer (LAK) cells to tumour deposits by bispecific monoclonal antibodies (bimAb) may be a way to improve adoptive immunotherapy. We developed a bimAb against adherent LAK (ALAK) cells and colon tumour CC531 in Wag rats. The bimAb was produced by somatic hybridization of two mouse hybridomas, one producing monoclonal antibodies (mAb) against CD8 (IgG2b, OX8), and the other producing mAb against a CC531-associated antigen (IgG1, CC52). A bimAb-producing clone was selected by an enzyme-linked immunosorbent assay with CC531 tumour cells. BimAb were purified from ascitic fluid by protein A affinity chromatography. Each of five pooled peak fractions was analysed by flow cytometry for the presence of bimAb. Most bimAb were found in a fraction that was eluted at pH 4.5 from protein A. FPLC analysis of this fraction revealed that no parental antibodies were present. The OX8 × CC52 bimAb greatly increased conjugate formation in vitro between ALAK cells and CC531. Results of ^{51}Cr-release assays with CC531 as target cells and ALAK cells as effector cells were not significantly different in the presence or in the absence of the bimAb. The methods we used here, a cell enzyme-linked immunosorbent assay and flow cytometry, are simple methods for development and purification of a bimAb when a functional selection method is not a priori available. The OX8 × CC52 bimAb we developed this way may increase in vivo tumour targeting of ALAK cells and thus augment antitumour effect in vivo.

Key words: Bispecific monoclonal antibody – Lymphokine-activated killer cell – Rat – CD8

Introduction

The efficacy of cellular immunotherapy using in-vitro-generated lymphokine-activated killer (LAK) cells in combination with interleukin-2 (IL-2) has been demonstrated in various murine tumour models [15]. The clinical application of this approach has met with some success in the treatment of renal cancer and malignant melanoma, but has been disappointing in other malignancies [16]. Yet in vitro observation show that almost all tumour cells are sensitive to LAK-cell-mediated lysis [9]. This discrepancy between in vitro and in vivo data is poorly understood. The number of LAK cells reaching the tumour site in vivo may be crucial for the antitumor effect. It has been shown in some animal models that LAK cells have the potential to migrate and infiltrate tumours [1], but other authors have demonstrated that LAK cells have aspecific homing properties [5]. Adoptive immunotherapy in combination with bispecific monoclonal antibodies (bimAb), directed against LAK and tumour cells, may increase the number of LAK cells at the tumour site, and/or prolong exposure time of tumour to these effector cells, and may augment antitumour effects. A condition for antigens on LAK and tumour cells to be suitable for bispecific targeting is that they have to be present on most of both cell populations.

The antibody CC52, directed against CC531 [2], a chemically induced rat colon cancer [4], was selected for one of the two specificities of the bimAb. Adherent LAK (ALAK) cells, which are known to be highly cytotoxic large granular lymphocytes [14], were cultured according to a protocol developed by Vujanovic et al. [21]. CD8, present on rat ALAK cells [10, 21], was chosen as antigen for the second specificity of the bimAb.

Here we describe the methods of development and purification of a bimAb for in vivo tumour targeting of rat ALAK cells.

Materials and methods

Tumour cell line. CC531 is a dimethylhydrazine-induced adenocarcinoma of the colon in Wag rats [4]. A cell line was established [2] and maintained by serial passage after trypsinization in culture medium, consisting of RPMI-1640 medium, Dutch modification, supplemented with 10% heat-inactivated fetal calf serum (both Gibco, Paisley, Scotland), 2 mM glutamine, 50 μg/ml streptomycin, and 50 U/ml penicillin.

Correspondence to: P. J. K. Kuppen, Department of Pathology, University of Leiden, P. O. Box 9603, 2300 RC Leiden, The Netherlands

CC531 cells were harvested with a solution of 0.25% EDTA and 0.05% trypsin in Hanks balanced salts solution for all experiments.

Generation of ALAK cells. ALAK cells were cultured according to a protocol described before [13, 21]. Briefly, spleens from 4- to 5-month-old male Wag rats (Harlan/CPB, Zeist, The Netherlands) were removed aseptically and crushed with the hub of a syringe in culture medium. B cells, macrophages, and monocytes were removed by incubation of the splenocytes for 1 h with nylon wool at 37° C in culture medium. The cells not adhering to nylon wool were collected and cultured in 75-cm^2 culture flasks (Greiner, Langenthal, Switzerland) each with 20 ml culture medium, supplemented with 1000 U/ml human recombinant IL-2 (EuroCetus, Amsterdam, The Netherlands), and 50 μM 2-mercaptoethanol, at a concentration of 2×10^6 cells/ml. After 24 h the culture supernatant was removed and filtered (0.2 μm). The cells non-adherent to plastic were removed by washing the culture flasks three times with 5 ml culture medium at a temperature of 37° C. The remaining (adherent) cells were cultured in the filtered conditioned medium for 5 days. The cells obtained showed high lytic activity against a broad spectrum of tumour cell lines, NK-resistant and NK-sensitive cell lines (data not shown).

Monoclonal antibodies (mAb). The OX8 [anti-(rat CD8)] IgG2b isotype switch variant was isolated in our Department from an OX8 IgG1 hybridoma (ECACC, Porton Down, Salisbury, UK) by limiting dilution. The anti-CC531 mAb, CC101 and CC52 (both IgG1), were also developed in our Department [2]. WT.1 {IgG2a, anti-(rat CD11a) [17]} was kindly provided by Dr. Miyasaka (Tokyo Metropolitan Institute for Medical Science, Tokyo).

Production of bimAb. The OX8 × CC52 quadroma was produced as described before [20]. Briefly, quadromas were generated by polyethyleneglycol fusion (PEG 1500, BDH Laboratory Supplies, Poole, UK) of parental-type cells with selection markers, and were seeded in 0.4% agar in hypoxanthine/aminopterin/thymidine medium (Gibco, Paisley, Scotland). Proliferating quadromas producing both parental-type antibodies, as indicated by positive immunohistochemistry on tumour and spleen, were tested for bi-isotypic antibody by a cell enzyme-linked immunosorbent assay (ELISA), and subcloned by limiting dilution.

Detection of bi-isotypic antibody by cell ELISA. CC531 cells were dispensed into 96-well V-bottom plates at 2×10^5 cells/well and incubated for 45 min at 4° C with 100 μl serial dilutions of quadroma culture supernatant. The cells were washed three times with 0.1 ml phosphate-buffered saline (PBS), supplemented with 0.5% bovine serum albumin (PBS/BSA), and incubated for 45 min at 4° C with a saturating amount of goat anti-(mouse IgG1), conjugated to horseradish peroxidase (HRP) or goat anti-(mouse IgG2b)-HRP (both from Nordic, Tilburg, The Netherlands), supplemented with 10% Wag rat serum. The cells were washed three times with PBS/BSA and incubated with *o*-phenylenediamine (Sigma, St. Louis, Mo., USA) for 10 min at room temperature in the dark. The staining reaction was stopped with 50 μl/well 2.5 M H_2SO_4. The plates were centrifuged (1100 rpm, 4 min) and 100 μl supernatant from each well was transferred to an ELISA plate. The absorbance (A) was measured at 492 nm by an ELISA reader. Quadromas, showing an A_{492} at plateau level with both anti-(IgG1)-HRP and anti-(IgG2b)-HRP of at least three times the background level (HRP conjugate only, $A_{492} = 0.00-0.25$) were scored as positive for production of bi-isotypic antibody.

Purification of bimAb. Ascitic fluid, produced in BALB/c F_1 mice, was three-times diluted with binding buffer (1.5 M glycine, 3 M NaCl, pH 8.9) and then centrifuged for 30 min at 10000 *g*. The supernatant was passed through a protein-A-Sepharose CL-4B column (Pharmacia, Uppsala, Sweden). Bound immunoglobulin was eluted using 0.1 M citrate buffer of pH 6 (fraction 1), 4.5 (fractions 2 and 3), and 3.0 (fractions 4 and 5). Each of the five peak fractions obtained was pooled and then dialysed against PBS. Finally, the fractions were concentrated by ultrafiltration (Diaflo XM50, Amicon, Danvers, Mass.) up to a protein concentration between 0.1 mg/ml and 1 mg/ml.

Flow-cytometric analysis of protein A fractions. Samples containing 5×10^5 ALAK cells were introduced into 12 × 75-mm plastic tubes in 0.1 ml PBS/BSA, and incubated for 30 min at 4° C with 2.5 μg antibody. The cells were washed twice with PBS/BSA, resuspended, and incubated for 30 min at 4° C with a saturating amount of both, fluorescein-isothiocyanate(FITC)-conjugated anti-(mouse IgG1) and phycoerythrin(PE)-conjugated anti-(mouse IgG2b) (both SBA, Birmingham, Alabama, USA). The cells were washed twice and analysed by flow cytometry for FITC and PE fluorescence. Dead cells were gated out by propidium iodide (1 μg/ml) exclusion.

FPLC analysis of OX8 × CC52. Samples containing about 1 mg protein were subjected to FPLC ion-exchange chromatography on mono-S Sepharose (Pharmacia, Uppsala, Sweden). Samples were dialysed against 40 mM sodium acetate buffer (pH 5.2) before application. Antibodies were eluted from the column with a linear gradient ranging from 0 up to 400 mM NaCl in the sodium acetate buffer. The elution profile was determined by registration of the absorbance of the eluate at 280 nm.

Conjugate formation assay. CC531 cells were incubated with a saturating amount of CC101 for 30 min at room temperature and then washed three times with PBS/BSA. Subsequently, the cells were incubated for 30 min with the anti-(mouse IgG1)-FITC and washed again three times with PBS/BSA. The CC531 cells coated with FITC-conjugated antibodies were designated FITC-CC531. ALAK cells were incubated with a saturating amount of WT.1 for 30 min at room temperature and then washed three times with PBS/BSA. Subsequently, the ALAK cells were incubated for 30 min with the anti-(mouse IgG1)-PE and washed again three times with PBS/BSA. The ALAK cells coated with PE-conjugated antibodies were designated PE-ALAK cells. Samples containing 6×10^5 of these PE-ALAK cells were incubated with 2.5 μg protein A fraction or parental mAb for 30 min at room temperature. The cells were washed twice with PBS/BSA and then mixed with 4×10^5 FITC-CC531 cells in 100 μl culture medium. The cell mixture was incubated for 30 min at 37° C. Finally, 900 μl PBS/BSA was added and the mixture was analysed by flow cytometry for the presence of conjugates showing both FITC and PE fluorescence. The percentage of CC531 cells conjugated with ALAK cells was calculated using quadrant analysis.

Chromium-51(^{51}Cr)-release assay. CC531 cells were washed once with culture medium and 10 μl packed cells (10^6 cells) was labelled for 1.5 h at 37° C with 100 μCi sodium [^{51}Cr]chromate in normal saline (Amersham, UK). Cells were then washed three times with culture medium. Samples of 100 μl ^{51}Cr-labelled target cells (5000 cells/well) and 100 μl effector cells at various effector-to-target (E : T) ratios were mixed in 96-well round-bottomed microtitre plates (Greiner, Langenthal, Switzerland). All tests were conducted in triplicate. Plates were incubated for 4 h or 19 h at 37° C, and centrifuted at 800 rpm for 5 min, after which 100 μl supernatant was removed and counted for release of ^{51}Cr, designated as experimental release (ER). Maximal release (MR) of ^{51}Cr-labelled cells was defined as the release obtained by the addition of 100 μl Triton X-100 with a concentration of 2% to 100 μl target cells. Spontaneous release (SR) was obtained by incubating 100 μl target cells with 100 μl culture medium without further additions. Specific release was calculated as follows:

$$\text{specific release (\%)} = \frac{\text{ER} - \text{SR}}{\text{MR} - \text{SR}} \times 100$$

Results

Development of OX8 × CC52 bimAb

BimAb was produced by fusing a hypoxanthine–guanine-phosphoribosyltransferase-deficient clone of a hybridoma producing anti-CD8 mAb (OX8, IgG2b) with a thymidine-kinase-deficient clone of a hybridoma producing anti-CC531 mAb (CC52, IgG1). Out of 60 quadroma clones tested, 12 were positive on spleen as well as on CC531 tumour tissue. These 12 clones were tested for the presence

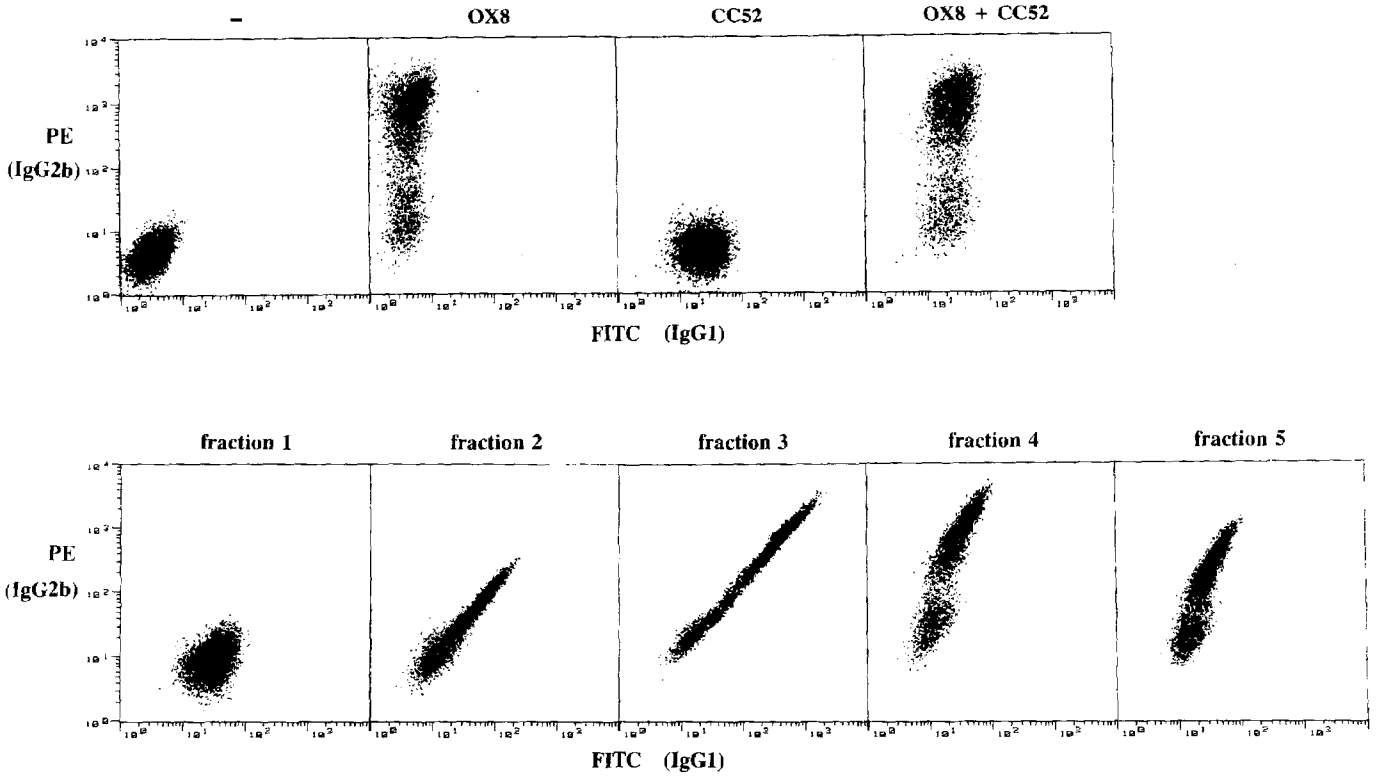

Fig. 1. Two-colour flow-cytometric analysis of OX8 × CC52 protein A fractions. Adherent lymphokine-activated killer (ALAK) cells were incubated with antibody or a protein A fraction as indicated above each diagram. ALAK cells were in all cases incubated with fluorescein-isothiocyanate(*FITC*)-labelled anti-(mouse IgG1) + phycoerythrin(*PE*)-labelled anti-(mouse IgG2b) as second step. Each diagram shows results from 10 000 cells

Table 1. Effect of cloning on stability of OX8 × CC52-producing quadroma[a]

Cloning number	Number of clones tested	Clones producing	
		Bi-isotypic antibodies (%)	Parental or no antibodies (%)
1	60[b]	17	83
2	10	60	40
3	10	60	40
4	10	80	20

[a] Production of bi-isotypic antibodies was determined in culture supernatant of quadroma clones by cell enzyme-linked immunosorbent assay (ELISA) as described in Materials and methods

[b] In this case only quadroma clones producing antibodies with specificity of both parental antibodies, as indicated by positive immunohistochemistry on CC531 tumour and spleen (12 out of 60), were tested for the presence of bi-isotypic antibodies by a cell ELISA

of antibodies in the culture supernatant consisting of the heavy chains from both parental mAb (bi-isotypic antibodies). This was done by application of an ELISA technique on CC531 cells, incubated with culture supernatant of the quadroma clones as described in Materials and methods. Of the 12 clones tested, 2 produced both parental mAb only, the other 10 produced bi-isotypic antibody. Thus, after fusion of the two parental hybridomas, only 17% (10/60) of the quadromas produced bi-isotypic antibodies. Of these 10 quadroma clones, 1 was chosen at random and subsequently subcloned three times by limiting dilution. After each round of subcloning 10 clones were tested for the presence of bi-isotypic antibody in a cell ELISA, and again, 1 clone, producing bi-isotypic antibody, was selected at random. The results of this cloning show that further subcloning after the first selection round did not improve the percentage of bi-isotypic-antibody-producing clones because it remained 60%–80% (Table 1). One quadroma clone was selected for production of ascitic fluid in mice after the third subcloning. Fractionation of the antibody from ascitic fluid was performed using protein-A-affinity chromatography and pH-gradient elution. Peak fractions were pooled and numbered 1–5.

Flow-cytometric analysis of protein A fractions

The OX8 × CC52 protein A fractions were analysed by two-colour flow cytometry for the presence of bi-isotypic antibody (Fig. 1) using ALAK cells. About 70% of the ALAK cell population was positive for CD8 expression and they all were slightly positive for the antigen recognized by CC52. OX8 antibody, as the parental mAb or part of a bimAb, was present in fractions 2, 3, 4 and 5. Fraction 1 contained only parental CC52 antibody. Fractions 2, 3, 4 and 5 contained bi-isotypic antibody as the PE-positive part of the ALAK cells was also positive for FITC-fluorescence. Fraction 3 appeared to contain the optimal combination of IgG1 and IgG2b. Sodium dodecyl sulphate/polyacrylamide gel electrophoresis analysis showed that equal

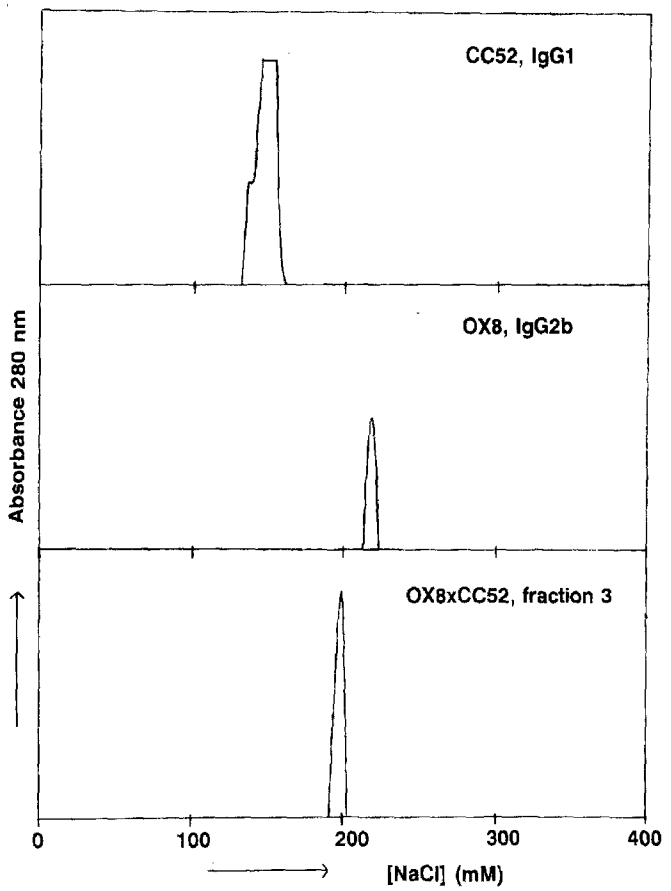

Fig. 2. FPLC analysis of OX8 × CC52 in fraction 3 and the parental antibodies OX8 and CC52. The antibodies were eluted from the mono-S Sepharose column with a linear gradient of NaCl. The concentration of NaCl in the gradient is indicated on the *x* axis and the absorbance of the eluate at 280 nm on the *y* axis

Table 2. Conjugate formation between adherent lymphokine-activated killer (ALAK) cells and CC531[a]

CC531-ALAK conjugates (%)							
Parental antibody			OX8 × CC52 protein A fraction				
–	OX8	CC52	1	2	3	4	5
18	30	36	28	69	88	46	43

[a] The percentage of CC531 conjugated with ALAK cells after preincubation of ALAK cells with parental mAb or a protein A fraction of OX8 × CC52, indicated in the table, was determined by flow cytometry as described in Materials and methods

amounts of the light and heavy chain of both parental mAb were present in this fraction (data not shown).

FPLC analysis of OX8 × CC52

Fraction 3 was tested by FPLC for the presence of parental antibodies. The parental CC52 antibody was eluted from the column at 148 mM NaCl (Fig. 2). The other parental antibody, OX8, was eluted at 220 mM NaCl. Fraction 3 was eluted as one peak from the column at 196 mM NaCl, and therefore appeared to contain no parental antibodies.

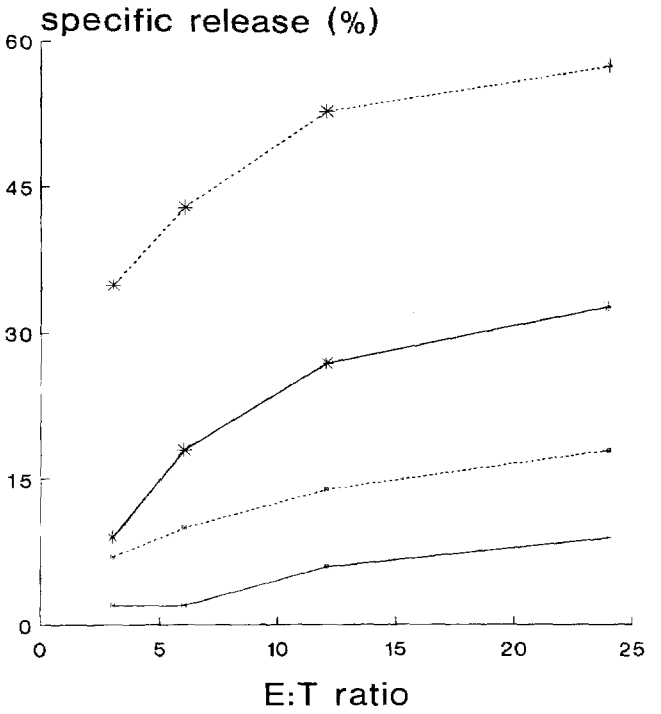

Fig. 3. Lysis of CC531 by ALAK cells. Specific release of ^{51}Cr was measured in the absence (———) or in the presence (– – – –) of 1000 U/ml interleukin-2. The incubation time was 4 h (■) or 19 h (*)

Conjugate formation between CC531 and ALAK cells

The cell ELISA and flow-cytometry analysis both measure bi-isotypic antibodies, binding with at least one Fab site to CC531 or ALAK cells. In order to determine whether the bi-isotypic antibody was functionally bispecific, i.e. contained two binding sites of different specificity, conjugate formation between CC531 tumour cells and ALAK cells was measured. Parental OX8 and CC52 mediated conjugate formation between ALAK and CC531 cells, slightly higher than the background (Table 2). Fraction 3 greatly increased conjugate formation. Fractions 2, 4, and 5 also increased conjugate formation between ALAK cells and CC531, but at a somewhat lower level than fraction 3. Fraction 1 did not mediate any conjugate formation higher than background level. Apparently, most functional bimAb was present in fraction 3.

Lysis of CC531 by ALAK cells

In 4-h ^{51}Cr-release assays lysis of CC531 was about 15% at an E:T ratio of 25:1 and fractionally higher when IL-2 (1000 U/ml) was present during the incubation period (Fig. 3). Maximal lysis (about 60% at E:T ratio 25:1) of CC531 was obtained after 19 h of incubation, in the presence of 1000 U/ml IL-2. In the absence of IL-2 the lysis of CC531 after 19 h of incubation was much lower: about

30% at E:T ratio 25:1. Addition of one of the protein A fractions had no effect on the lysis level of CC531. We tested a concentration range of 0.04–4 μg/ml of all fractions, in the absence and presence of IL-2 after 4 h and 19 h of incubation, but observed no significant difference in lysis of CC531 under any conditions in the presence or in the absence of one of the fractions (data not shown).

Discussion

Adoptive immunotherapy of LAK cells in combination with bimAb, directed against LAK and tumour cells, may augment antitumour effects. Some studies have reported the development of bimAb against human CD16 and tumour antigens [6, 7, 18]. In combination with LAK cells these bimAb were able to prevent tumour outgrowth in a Winn-type assay in nude mice [18]. However, this is a rather artificial in vivo experiment as tumour and effector cells are mixed and injected at the same time. For evaluation of the effect of bimAb on tumour targeting of LAK cells, syngeneic animal models are necessary in which situations can be tested with established tumours. Therefore, we developed a rat tumour model to evaluate the effect of bimAb on the therapeutic effect of adoptive immunotherapy with LAK cells [12, 13]. We have made a bimAb with antigen specificity for rat ALAK cells and the rat colon tumour CC531. The bimAb is directed against CD8 on rat ALAK cells. We chose this antigen because most rat ALAK cells express CD8 [10, 21]. The most important function of a bimAb may be to serve as an anchor for LAK cells, specifically at the site of a tumour. Therefore, probably any antigen expressed by LAK cells can be used. For bispecific targeting of human ALAK cells, for example, CD56 may be very suitable as most of these cells express this antigen [14].

For the development of a bimAb it is necessary to screen quadroma clones for its production. Contrary to bimAb-mediated targeting of T cells with anti-(T cell receptor) × antitumour bimAb [2, 8, 20], triggering to induce lysis is not a prerequisite for LAK cells as these cells are already activated. Therefore, the method used for selection of an anti-(T cell) × antitumour bimAb, which measures the bimAb-mediated enhancement of tumour cell lysis in ^{51}Cr-release assays [20], could not be used to select a quadroma producing the bimAb we developed in this study. We produced a bimAb from parental antibodies of two different isotypes, to enable selection of bimAb-producing quadromas by testing for the presence of bi-isotypic antibody by a cell ELISA. As antibodies of different isotype have different affinities for protein A, it also simplified the purification procedure.

The number of clones, after fusion of the parental hybridomas, producing antibodies reacting with both tumour tissue and spleen was rather low (12 out of 60), although most of them (10 out of 12) produced bi-isotypic antibodies. Subsequent subcloning of a bi-isotypic-antibody-producing quadroma resulted in 60%–80% clones producing bi-isotypic antibodies. Apparently some instability is inherent in this quadroma of OX8 × CC52. Two-colour flow-cytometric analysis clearly showed the bi-isotypic nature of the OX8 × CC52 antibody we have developed. The purified OX8 × CC52 was not only bi-isotypic but also a functional bimAb, i.e. it had two binding sites, one with specificity for CD8 on ALAK cells and the other for an antigen on CC531 tumour cells, as conjugate formation between ALAK cells and CC531 was greatly increased. Analysis by FPLC revealed that no parental antibodies were present in the purified fraction as the elution profile demonstrated only one peak. Furthermore, the OX8 × CC52 bimAb was eluted from the mono-S Sepharose column at a NaCl concentration in between those of both parental antibodies.

We performed in vitro experiments to study the effect of the bimAb OX8 × CC52 on the interaction between ALAK and CC531 cells with respect to conjugate formation and lysis. CC531 appeared to be rather insensitive for ALAK lysis. A long incubation period and the presence of IL-2, most likely to keep the ALAK cells in an activated state, were necessary for optimal lysis. However, the contact between the CC531 tumour cells and ALAK cells in ^{51}Cr-release assays apparently is not a limiting factor as the bimAb had no effect at all on the lysis level. We have shown before that the OX8 antibody was able to redirect killing of the Fc-receptor-positive tumour cell line P815 by ALAK cells, resulting in enhanced lysis [11]. Our results demonstrate that triggering of CD8 on ALAK cells by an antibody with one Fab fragment recognizing this antigen is not possible as the bimAb OX8 × CC52 did not increase lysis of CC531 by ALAK cells. This is analogous to the situation of LAK cells with bimAb against CD16 where LAK-mediated tumour lysis also was generally not higher in the presence of these antibodies [6, 18].

Currently we are developing two other bimAb, both directed against CC531 and with CD2 or NKR-P1 as ALAK specificity. Both of these ALAK antigens have been shown to be activation structures [3, 11] and are therefore probably also suitable for targeting of ALAK cells. NKR-P1 especially is interesting as this natural-killer(NK)-related structure [19] is expressed by all ALAK cells of the rat [3].

In conclusion, we have shown here that cell ELISA and flow cytometry are simple methods when no functional assay for detection of bimAb is available. The purified preparation of OX8 × CC52 greatly increased conjugate formation between target and effector cells. Therefore, this OX8 × CC52 antibody is suitable for in vivo targeting studies of ALAK cells and may increase antitumour effects of ALAK cells in vivo rat tumour models.

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