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Tumour cell killing using chemically engineered antibody constructs specific for tumour cells and the complement inhibitor CD59

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SUMMARY

Immunotherapy using MoAbs is inefficient due to limited activation of human effectors by mouse antibodies and multiple protective mechanisms available to host cells against autologous complement. We have used chemically engineered antibody constructs and human complement *in vitro* to specifically target and kill neoplastic B lymphoid cells (Raji). Fab′γFcγ_2 chimaeric antibody (specific for human CD37) was used to activate the classical pathway of human complement on Raji cells, whilst CD59 was neutralized using one of two different bispecific F(ab′γ)$_2$ antibody constructs which contained both cell-targeting (anti-CD19 or anti-CD38) and CD59-neutralizing moieties. When either bispecific construct was used to neutralize CD59, 15–25% of cells were lysed. If CD55 was also neutralized using specific antibody, Raji cells were efficiently killed (70% lysis). When added to a mixture of target (Raji) and bystander (K562) cells, one bispecific antibody (anti-CD38 × anti-CD59) could be specifically delivered to Raji, avoiding significant uptake on CD59-expressing bystander cells (K562). The second bispecific antibody (anti-CD19 × anti-CD59) bound equally well to either cell type. Cell-specific targeting was dependent upon combination of a low-affinity anti-CD59 Fab′γ with a high-affinity anti-tumour cell Fab′γ. When Raji and K562 cells were mixed and incubated with a combination of the engineered constructs and anti-CD55 antibodies, Raji cell lysis (30–40%) was observed in the absence of K562 killing. We propose that combinations of these constructs may be of use for treatments such as *ex vivo* purging of autologous bone marrow or *in vivo* targeting of tumour cells.

Keywords complement monoclonal antibodies CD59 B lymphocytes immunotherapy

INTRODUCTION

MoAbs have been used in the therapy of human tumours, particularly in the treatment of malignancies of the haematopoietic system, where cells may be circulating and more accessible to treatment [1,2]. Antibodies have been used to recruit the patient's own effector systems, leading to target cell death via antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-mediated killing. However, therapeutic success has been limited for various reasons, such as poor activation of human effector systems by mouse antibodies, generation of a human anti-mouse antibody (HAMA) response, short *in vivo* half-life and protection of human cells from homologous complement attack by inhibitors such as membrane cofactor protein (MCP; CD46), decay-accelerating factor (DAF; CD55), complement receptor 1 (CR1; CD35) and CD59. Immunotherapy has also been used *ex vivo*, particularly for purging of autologous bone marrow in the treatment of leukaemia [3–6]. *Ex vivo* purging procedures include treatment of cells with mouse MoAbs and rabbit serum complement; frequently a 'cocktail' of antibodies is used with specificities for a range of antigens on the target cell surface. The use of rabbit complement overcomes to some extent homologous restriction of activation on human cells. However, there are also problems associated with the use of complement from another species, such as batch-to-batch variation, background toxicity and immunogenicity. This would be avoided if autologous serum could be used as the source of complement.

CR1 and DAF are decay-accelerating factors and act by destabilizing C3 and C5 convertases formed through activation of either the alternative or classical pathways [7–10]. CR1 also acts as cofactor for cleavage and inactivation of C3b and C4b by factor I, as does MCP [7,11]. CD59 differs from the other three membrane-bound complement inhibitors, in that it inhibits the terminal pathway of complement [7,12]. It acts at the C5b-8 stage by preventing binding and subsequent polymerization of C9 in the membrane. The aim of our work was to target complement-mediated killing to

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tumour cells and, by blocking CD59 on the target cell surface, to improve the efficiency of killing of these cells by antibody and homologous complement. We used chemical engineering to generate antibody constructs specifically designed either to target complement activation to neoplastic B cells (chimaeric Fab′γFcγ_2; FabFc_2) or to block the function of CD59 on these cells (F(ab′γ)$_2$ bispecific antibody; bsAb). The chimaeric construct contains Fc moieties derived from human IgG1. The cell-targeting moieties of both the chimaeric and bispecific constructs are derived from either mouse or rat MoAbs and are specific for B cell antigens (CD19, CD37 or CD38). Provided that haematopoietic stem cells are not affected by the treatment, it is possible to target leukaemic cells using antibody to antigens that are also expressed on non-malignant cells. Populations of normal cells can be renewed from antigenically dissimilar precursor cells in the bone marrow.

Here we present data from *in vitro* studies using the Burkitt lymphoma line Raji+3 as a target cell line. Previous work has demonstrated that CD59 and DAF are of paramount importance for protection of these cells against complement attack [13]. Whilst MCP is also expressed by this cell line, it has a limited protective role against classical pathway-mediated lysis [13–15]. We have demonstrated that cells coated with a combination of complement-activating and CD59-blocking constructs can be efficiently lysed using homologous complement provided that DAF is also neutralized on the cell surface. We have assessed specificity of delivery of bispecific constructs to other CD59-expressing cells (K562), analysed the consequent specificity of cell killing, and demonstrated that appropriate pairing of Fab moieties into bispecific constructs led to efficient and specific delivery of construct to the target cell. We propose that such constructs may be used *ex vivo* (and possibly *in vivo*) in the treatment of haematopoietic malignancies to specifically eradicate tumour cells.

MATERIALS AND METHODS

Cells

Raji and K562 cell lines were obtained from the European Collection of Animal Cell Cultures (Porton Down, UK). The Raji line used in these experiments was a subline of Raji previously described by us, Raji+3 [13]. As B cell lines have previously been demonstrated to lose expression of glycosyl phosphatidylinositol (GPI)-anchored complement inhibitors [13,16], this line was selected to ensure that the GPI-linked complement inhibitors CD59 and DAF were expressed on the cell surface. MCP, but not CR1, was also expressed by cells of this line.

Antibodies

MoAbs were obtained from the following sources. BRIC216 and BRIC110 (anti-DAF; CD55) from International Blood Group Reference Laboratory (IBGRL; Bristol, UK); BRIC229 (anti-CD59) from IBGRL. All BRIC antibodies were used as tissue culture supernatant. YTH53.1 (anti-CD59) was purified from ascites by protein G affinity chromatography, the cell line producing YTH53.1 was obtained from H. Waldmann (Oxford, UK). The following IgG were purified using protein A affinity chromatography and the hybridomas were obtained from the following sources: 5.18 (anti-human IgG Fc) was produced in our laboratory (Cardiff, UK); MEM43 (anti-CD59) was obtained from V. Horejsi (Prague, Czech Republic); RFB9 (anti-CD19) was produced in the Department of Immunology, Royal Free Hospital (London, UK); WR17 (anti-CD37) was produced in the Wessex Regional Immunology Unit (Southampton, UK); AT13/5 (anti-CD38) was produced in the Tenovus Research Laboratory (Southampton, UK). Fluorescently labelled antibodies (Fab_2 fragments) (goat anti-mouse (GAM)-PE and rabbit anti-mouse (RAM)-FITC) were obtained from Dako Ltd (High Wycombe, UK).

All anti-CD59 and anti-DAF antibodies mentioned above have blocking activity. However, in our hands, total inhibition of DAF function was only achieved when BRIC110 and BRIC216 were used in combination. When 'anti-DAF antibodies' is specified in the text, this indicates use of 10 μg/ml of each anti-DAF antibody.

Preparation of monospecific Fab_2 fragments and bispecific Fab_2 constructs

Chemically engineered bispecific antibody constructs were produced as described previously [17]. Briefly, monospecific Fab_2 fragments (msAb) of mouse antibodies RFB9, AT13/5 and MEM43 were prepared first by limited proteolysis of antibody IgG by pepsin. YTH53.1 was digested with *Staphylococcus aureus* V8 protease. To prepare bispecific Fab_2 (bsAb), msAb from each of the selected partners were reduced with DTT to yield monomeric Fab$(SH)_5$ (mouse antibodies) or Fab$(SH)_4$ (rat). One of the Fab was reacted with the linker *o*-phenylenedimaleimide to yield Fab(maleimide). The Fab$(SH)_n$ and Fab-maleimide were allowed to react to form bispecific Fab_2 which was separated from other products, principally Fab_3, by chromatography on Superdex 200.

Preparation of chimaeric $FabFc_2$

Fab_2 was prepared by digestion of the mouse IgG2a antibody WR17, and Fab$(SH)_5$ was then derived by reduction with DTT. Fc-maleimide, in which a free maleimide group is linked to a hinge-region cysteine in Fc derived from human IgG1 by digestion with papain, was prepared as described previously [17]. The Fab and Fc, at a molar ratio of 1:2·5, were allowed to react and the predominant product, $FabFc_2$, was then separated by recycling chromatography on Superdex 200.

Fluorescent staining

In order to stain cell surface antigens, cells were incubated at 4°C with specific antibody at 10 μg/ml (BRIC229, BRIC216, RFB9, AT13/5 (Fab fragment)) or WR17 tissue culture supernatant (50%), followed by 5 μg/ml RAM-FITC. Cells were fixed with 2% paraformaldehyde in PBS and fluorescence was analysed using a Becton Dickinson FACScan (Oxford, UK). For analysis of construct binding to a single cell type, cells were incubated with various concentrations of construct for 45 min at room temperature ($FabFc_2$) or 90 min at 4°C (bsAb). $FabFc_2$ was detected using 5.18 (anti-human IgG Fc; 5 μg/ml) and RAM-FITC (5 μg/ml). BsAb was detected using GAM-PE (10 μg/ml). Fluorescence was analysed using a Becton Dickinson FACScan. In order to study specificity of construct binding, K562 cells were first fluorescently labelled with PKH2 membrane dye according to the manufacturer's instructions (Sigma, Poole, UK). To assess bsAb or msAb binding to a mixture of cells, K562 (labelled with PKH2) and Raji+3 (unlabelled) were mixed (5×10^5/ml of each cell type) and incubated on ice in complement fixation diluent (CFD; Oxoid Ltd, Basingstoke, UK) containing 0·1% gelatin (GVB), for 90 min with the antibody fragments. Bound antibody was stained with GAM-PE (10 μg/ml) and analysed on the FACScan. Two populations of cells were

Table 1. Antibody specificity and isotype

Antibody	Specificity	Isotype
BRIC229	CD59	Mouse IgG2b
MEM43	CD59	Mouse IgG2a
YTH53.1	CD59	Rat IgG2b
BRIC216	DAF (CD55)	Mouse IgG1
BRIC110	DAF (CD55)	Mouse IgG1
AT13/5	CD38	Mouse IgG1
RFB9	CD19	Mouse IgG1
WR17	CD37	Mouse IgG2a

BsAb1 (anti-CD59 × anti-CD38) is MEM43 × AT13/5). BsAb2 (anti-CD59 × anti-CD19) is YTH 53.1 × RFB9.)

analysed: FL1 bright (K562 cells, PKH2 dye detection) and FL1 dim (Raji+3 cells). Binding of construct was assessed using the FL2 axis (GAM-PE detection).

Killing assay using propidium iodide

Cells were washed and sensitized by incubating cells (10^6/ml) at room temperature with constructs or other antibodies as indicated in the figures and legends. Cells were then washed in GVB, resuspended to the original volume, and incubated with serum at 37°C for 1 h. This serum had previously been incubated on ice for 1 h with 10^7 Raji+3 cells per ml of serum to remove any naturally occurring antibodies. To assess cell death, cells were transferred onto ice and propidium iodide (4 μg/ml final concentration) was added to each sample. Viability was assessed immediately using a FACScan. In the absence of $FabFc_2$, no killing could be achieved by 'sensitizing' cells with any combination of the antibodies (BRIC110, BRIC216 or BRIC229), indicating that these antibodies were not complement activating (data not included).

Killing assay using calcein AM

All dead cells in an incubation are fluorescently labelled by propidium iodide. Therefore when analysing cell killing in a mixture of cells, one population of dead cells cannot be distinguished from another. In order to analyse specificity of killing, either K562 or Raji+3 cells were labelled with an intracellular fluorescent dye and two parallel experiments were carried out. Either labelled Raji+3 cells were incubated with unlabelled K562 to assess Raji+3 cell death (as described below), or labelled K562 were incubated with unlabelled Raji+3 to assess K562 cell death (under identical conditions). Cells were labelled with the green fluorescent probe calcein AM (Molecular Probes, Eugene, OR) by incubating 2×10^6 cells/ml with calcein AM (1·3 μg/ml) at 37°C for 30 min. Cells were then washed, resuspended in GVB, and sensitized as described in the previous section. Following sensitization, cells were washed, resuspended to 10^6 cells/ml and incubated for 1 h at 37°C with serum containing $FabFc_2$ (5 μg/ml). Cells were centrifuged once and analysed on a Becton Dickinson FACScan. Lysed cells have a damaged plasma membrane and consequently leak the fluorescent dye from the cytoplasm. These cells show as a distinct 'dim' population of cells on FACScan analysis, whereas live cells are 'bright'. Both dim and bright cells are easily distinguished from unlabelled cells. Percentage cell death can be calculated for test and control populations as follows: percentage cell death = (number of dim cells × 100)/(total number of bright + dim cells).

RESULTS

Target cell killing using antibody constructs

Complement was activated on the surface of Raji+3 cells using the chimaeric construct, $FabFc_2$. The Fab moiety was specific for CD37, enabling targeting to Raji+3 cells, whilst the Fc arms were derived from human IgG1 to promote activation of human complement. Two bsAb were also prepared which had the potential to block CD59 on target cells. These were used in combination with $FabFc_2$. The first, bsAb1, had specificity for CD38 and CD59, and the second, bsAb2, had specificity for CD19 and CD59 (see Table 1 for antibody details). Data in Fig. 1 demonstrate that $FabFc_2$ can activate complement on Raji+3 cells and that both bsAb can block CD59 function. As expected, $FabFc_2$ alone did not induce cell lysis, as all three complement inhibitors were active on the cell surface. Blocking only DAF caused a small enhancement of cell

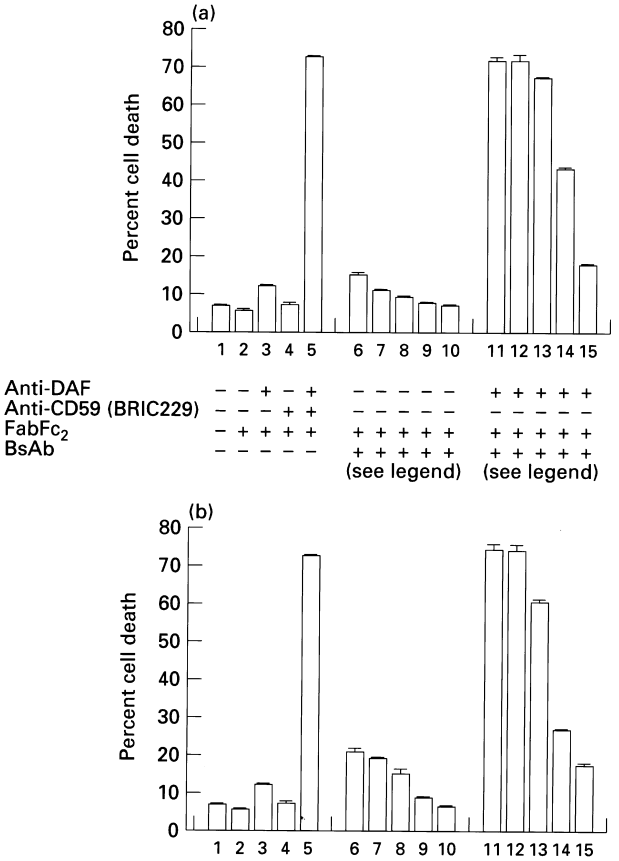

Fig. 1. Tumour cell killing (Raji+3) using bispecific antibody constructs (anti-tumour × anti-CD59). Cells were sensitized for 70 min with $FabFc_2$ (10 μg/ml) and other antibodies or constructs as indicated. Neutralizing anti-decay-accelerating factor (DAF) antibodies (BRIC110 and BRIC216) were used at 10 μg/ml and anti-CD59 (BRIC229) at 2 μg/ml. BsAb1 (a) or bsAb2 (b) were used at 2·5, 0·5, 0·1, 0·02 or 0·004 μg/ml (bars 6–10 and 11–15, respectively). Cells were incubated with normal human serum (NHS) at a final concentration of 1:8 and killing was assessed using propidium iodide uptake as a marker for cell death. Results are means of duplicates ± range.

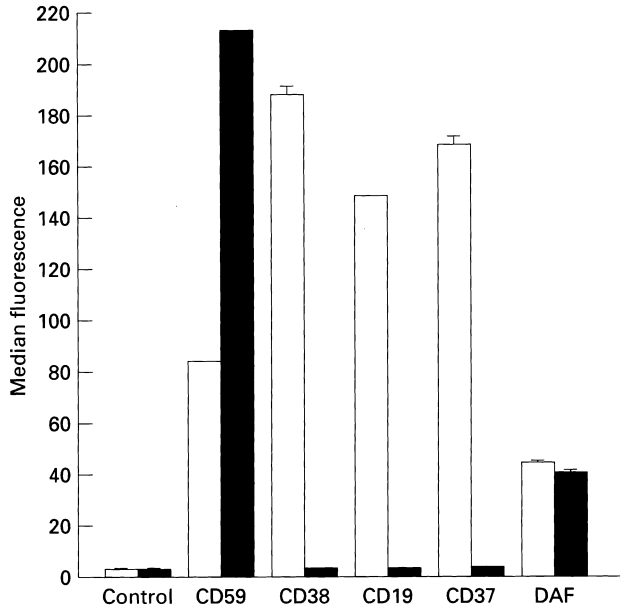

Fig. 2. Cell surface expression of CD59, CD38, CD19, CD37 and decay-accelerating factor (DAF) on Raji+3 cells (□) or K562 cells (■). Cells were stained using BRIC229 (anti-CD59), AT13/5 (anti-CD38), RFB9 (anti-CD19), WR17 (anti-CD37) or BRIC216 (anti-DAF) first antibody, followed by rabbit anti-mouse (RAM)-FITC. Control cells were stained with second antibody only. Binding was assessed using flow cytometry. Results are means of duplicates ± range.

lysis (< 10% increase), whereas blocking CD59 alone (using BRIC229 antibody) had no effect. If both CD59 and DAF were blocked on these cells (using BRIC antibodies) > 70% of cells were lysed (bar 5). This showed that $FabFc_2$ chimaeric antibody activated human complement on these cells, and demonstrated the need to neutralize both CD59 and DAF on the surface of Raji+3 cells in order to attain high levels of cell death. Both bispecific constructs demonstrated a weak enhancement of cell lysis (bars 6–10) when used at higher concentrations (> 0·1 μg/ml). Killing was greatly amplified by also blocking DAF (bars 11–15), indicating that complement was efficiently activated on these cells and that CD59 had been neutralized.

Specificity of construct binding

The constructs have various specificities including CD59, CD37, CD38 and CD19. Raji+3 cells expressed CD38, CD37 and CD19, whereas K562 expressed none of these antigens (Fig. 2). Whilst both cell lines expressed similar levels of DAF, K562 cells expressed more than twice the amount of CD59, making them ideal cells to use for analysis of construct binding to bystander cells. Chimaeric $FabFc_2$ bound to Raji+3 cells which expressed CD37, but not to K562 ($CD37^-$) (Fig. 3a). Univalent binding of bsAb1 (anti-CD38 × anti-CD59) to K562 ($CD38^-$, $CD59^+$) was very low (Fig. 3b), indicating a low-affinity binding through the anti-CD59 Fab (MEM43). Binding of the same construct to Raji+3 cells ($CD38^+$, $CD59^+$), was much higher, suggesting that the abundant binding of bsAb1 to Raji+3 cells was mediated through the anti-CD38 Fab, which is known to be of high affinity (our unpublished data). In contrast, bsAb2 (anti-CD19 × anti-CD59) bound equally to both Raji+3 ($CD19^+$) and K562 cells ($CD19^-$), indicating that the binding through this anti-CD59 Fab (YTH53.1) was with a higher affinity than MEM43 (Fig. 3c). The presence of the anti-CD19 Fab did not significantly increase binding of bsAb2 to Raji+3 cells.

Due to differences in binding of the detection antibody (FITC- or PE-conjugated) to the different constructs, it is not possible to directly compare binding of different constructs on the same cell surface. This is particularly important in the case of bsAb2, where one Fab moiety (YTH53.1) is of rat origin, and whilst GAM IgG detection antibody cross-reacts with this species Fab, the resulting staining intensity is less than that obtained with mouse Fab. Hence, levels of staining of bsAb1 appear higher than with bsAb2 (also evident in Fig. 5).

Whilst binding of bsAb1 was demonstrated only at low levels on K562 cells, it was clear that when held on the surface of Raji+3, which express both CD59 and CD38, the anti-CD59 arm (derived from MEM43) neutralized CD59 function (Fig. 1). The anti-CD59 Fab contained within bsAb1 was also demonstrated to bind CD59 on double-positive ($CD59^+$, $CD38^+$) cells, as evidenced by its ability to block binding of the parent antibody, MEM43 (Fig. 4). BsAb1 bound to Raji+3 cells at 0·5 μg/ml and blocked binding of biotinylated MEM43 IgG to CD59 (Fig. 4a) (anti-CD38 Fab alone had no effect on subsequent binding of MEM43 IgG to the cells (data not shown)). In contrast, bsAb1 bound weakly to K562 cells (Fig. 3b), and was therefore not able to block binding of the divalent parent antibody to CD59 on K562 cells (Fig. 4b).

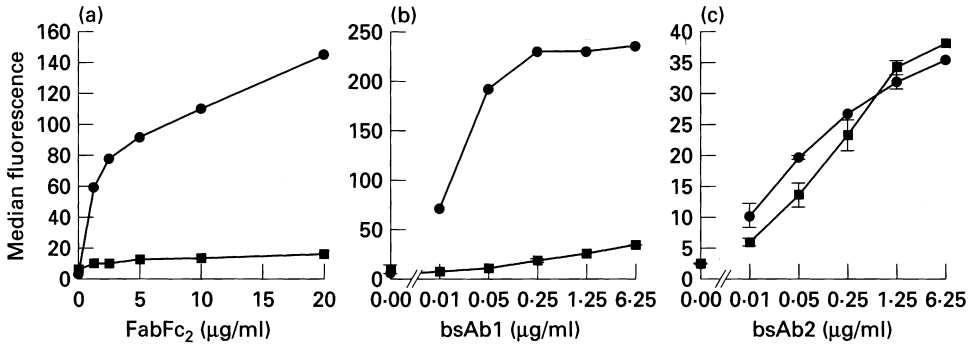

Fig. 3. Binding of antibody constructs to Raji+3 (●) or K562 (■) cells. (a) Cells were incubated with $FabFc_2$, 5.18 (anti-human IgG Fc) and rabbit anti-mouse (RAM)-FITC. Binding of bsAb1 (b) or bsAb2 (c) was assessed by incubating cells with bispecific antibody and goat anti-mouse (GAM)-PE. Binding was measured using flow cytometry. Results are means of duplicates ± range.

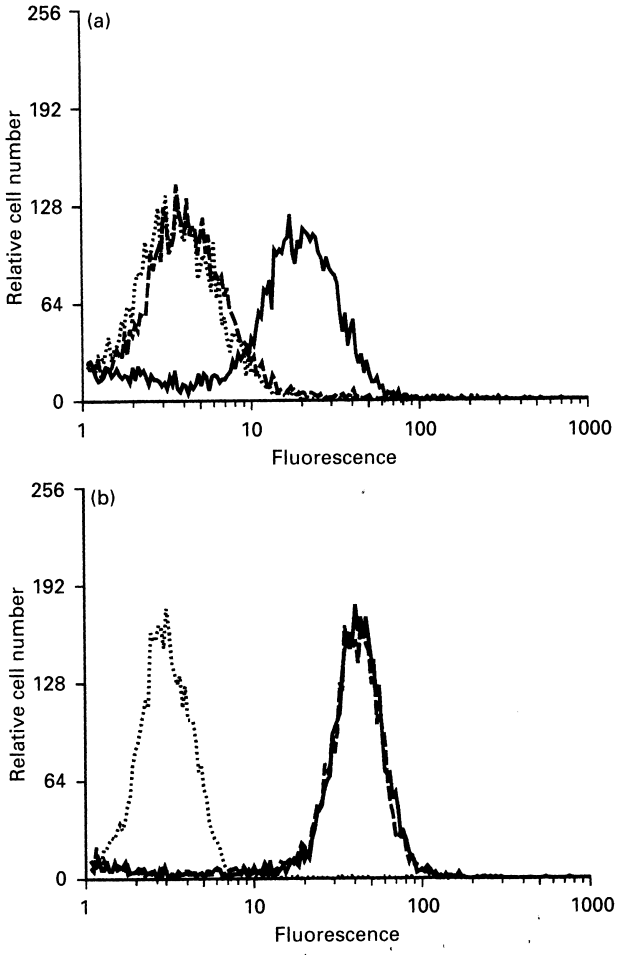

Fig. 4. Blocking of biotinylated MEM43 IgG binding to (a) Raji+3 or (b) K562 cells using bsAb1. Raji+3 or K562 cells were incubated in the absence (————) or presence (- - - - -) of 0·5 μg/ml bsAb1. Cells were then incubated with 5 μg/ml biotinylated MEM43 IgG and 10 μg/ml streptavidin-FITC before analysis by flow cytometry. Control cells (·····) were incubated with streptavidin-FITC only.

Specificity of construct delivery

The binding of bsAb and monospecific (anti-CD59) Fab_2 fragments (msAb) to either Raji+3 or K562 cells present in a mixture of cells was studied using PKH2 fluorescent membrane dye to 'tag' the K562 cells, as described in Materials and Methods. Figure 5a,b demonstrates binding of the msAb to cells in the mixture. K562 express higher levels of CD59 and consequently bound more anti-CD59 msAb (both MEM43 and YTH53.1), as expected. However, the presence of a B cell-specific moiety in the bispecific construct significantly altered the distribution of antibody (Fig. 5c,d). Using either anti-CD38 (bsAb1) or anti-CD19 (bsAb2) Fab arms increased binding of construct to the Raji+3. This effect was particularly marked with bsAb1, where Raji+3 cells bound 12 times as much construct as the bystander cells (at 100 ng/ml). A targeting effect of bsAb2 was evident at low concentrations of construct (20 ng/ml or 4 ng/ml), where Raji+3 cells bound more than twice as much bsAb2 as K562 cells. These data demonstrate that targeting (or distribution of bsAb amongst different cells) is crucially dependent on the combination of Fab moieties in the construct.

Specificity of killing using constructs

In order to analyse specificity of killing in an incubation containing more than one cell type, we used the intracellular green fluorescent probe calcein. Data in Fig. 6 demonstrate that by using $FabFc_2$ to activate complement, killing of target cells (Raji+3) was achieved in the absence of K562 lysis. As in Fig. 1, neutralization of DAF enhanced cell lysis slightly (20% cell death). If CD59 was also neutralized using either msAb or bsAb, killing was further enhanced (30–40% cell death). Levels of Raji+3 killing were lower with bsAb2 than with bsAb1. This may reflect binding of bsAb2 to the bystander cells (K562) resulting in a low concentration of construct on the surface of the target cell (Raji+3).

It should be noted that for various reasons it is not possible to directly compare (w/w) the blocking activities of monospecific and bispecific antibodies. For example, monospecific antibodies have two arms specific for CD59, which can enhance the blocking power of this antibody compared with the bispecific construct. The avidity of binding of the monospecific antibody may also be significantly different from that of the bispecific antibody (particularly in the cases of binding of bsAb1 or msAb (MEM43) to K562 cells). Another consideration is that cross-linking two CD59 (msAb) may have different consequences to the cell compared with linking CD59 to a different antigen (using bsAb).

DISCUSSION

Various chemically engineered antibody constructs have been used *in vivo* for treatment of B cell lymphoma. $FabFc_2$ constructs have been shown to be effective at reducing circulating tumour cell burden in patients [18], and bispecific Fab_2 immunotoxin constructs (anti-tumour × anti-saporin) have been used successfully *in vivo* to target saporin to tumour cells in a guinea pig L_2C tumour model [19]. Humans with lymphoma have also been treated with similar constructs (to deliver saporin) and have sometimes shown marked clinical improvements [20,21]. Several other applications of chemically engineered antibodies have been assessed *in vitro*, such as the use of trispecific Fab_3 to redirect resting cytotoxic T cells by cross-linking several antigens on the T cell (e.g. CD2 and CD3) with a tumour cell antigen (CD37) [22]. Here we describe the chemical engineering of antibodies to generate constructs that can target tumour cells *in vitro* and improve efficiency of lysis by homologous complement. We used a combination of a killing construct (univalent chimaeric $FabFc_2$) that both recognized the target cell surface and activated human complement, and bispecific antibody containing a cell targeting arm and a CD59-blocking arm. We have demonstrated that these complement-activating and CD59-blocking constructs can be specifically targeted to tumour cells and used successfully to activate homologous complement and block CD59 function on the target cell surface, rendering the tumour cells sensitive to lysis by homologous complement.

The $FabFc_2$ construct has several important design features. First, the presence of more than one Fc moiety on the chimaeric construct facilitates formation of a lethal array on the target cell surface, as cross-linking of at least two Fc is required to activate the C1q component of the classical pathway. Second, the construct is univalent. By reducing antigenic modulation (the aggregation and internalization of surface antigen which follows cross-linking by antibody) univalent antibodies have been shown to lead to enhanced complement lysis [23–25]. Finally, Fc fragments derived from human IgG are incorporated into the construct. 'Humanization'

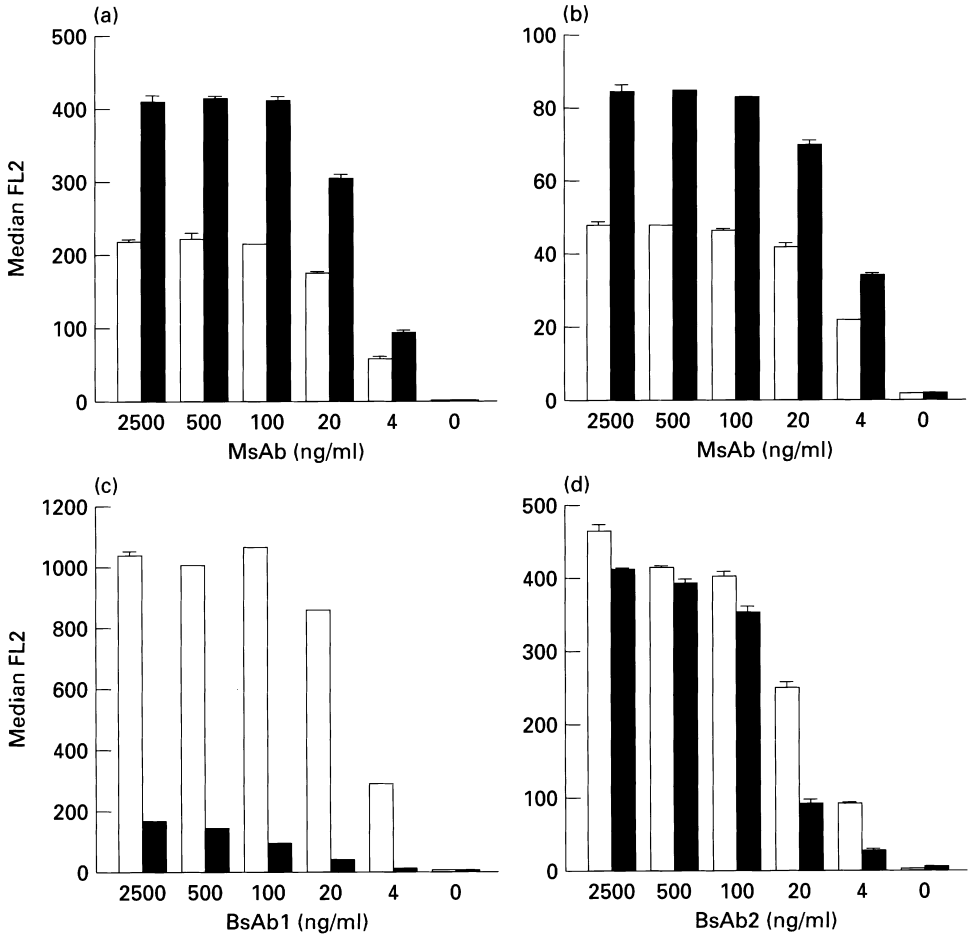

Fig. 5. Specificity of msAb and bsAb binding to a mixture of Raji+3 and K562 (PKH2 labelled) cells. Cells were incubated with the indicated concentrations of (a) MEM43 msAb (anti-CD59), (b) YTH53.1 msAb (anti-CD59), (c) bsAb1 (anti-CD59 × anti-CD38), (d) bsAb2 (anti-CD59 × anti-CD19). Construct binding to Raji+3 (□) or K562 (■) was analysed as described in Materials and Methods. Results are means of duplicates ± range.

of therapeutic antibodies is important both for the extension of their *in vivo* half-life and for efficient activation of human effectors. The presence of immunogenic murine Fab fragments will potentially reduce the utility of these constructs *in vivo*. However, future work will involve the development of totally 'humanized' recombinant constructs by complementarity determining region (CDR) grafting techniques [26], or production of recombinant human Fab using phage display technology to screen a library containing human antibody genes [27–29].

Cell-specific targeting of the bispecific inhibitory constructs is essential in order to reduce 'mopping up' of construct by other cells, as CD59 is expressed on many cell types including all circulating cells, epithelial cells and endothelial cells. Non-specific binding would lead to dilution of the construct on the target cell membrane and possible lysis of innocent bystander cells. We have tested binding characteristics and lytic efficiencies of two different anti-CD59 bispecific antibodies *in vitro*. As both CD59 and DAF have to be targeted and inhibited on the cell membrane in order to kill Raji+3 cells, anti-DAF antibodies were used to 'develop' cell killing in the assays. When these bispecific constructs were used on cells on which DAF had been blocked, treatment with human serum resulted in efficient lysis of the cells, whereas in the absence of targeted blocking of CD59, lysis was minimal (Fig. 1).

Our data demonstrate that the bispecific construct used to neutralize complement inhibitors must have very specific properties. It must have much lower affinity for the complement regulator than for the anti-cell component, but must retain potent neutralizing activity. By using Fab partners of differing affinities for antigen it was possible to specifically deliver bispecific antibody (bsAb1) to target cells (Raji+3), avoiding significant uptake on K562 cells expressing CD59 (Figs 3 and 5). The avidity of binding of such a construct to a double-positive cell greatly exceeds that of binding to a cell expressing CD59 alone. BsAb1 is thus more suited to therapy *in vivo* (or *ex vivo*) than is bsAb2, as bsAb1 can be delivered specifically, maximizing the concentration of construct on the target cell surface (Fig. 5).

Our strategy has been to block CD59, the major inhibitor of the membrane attack complex, in order to render tumour cells more susceptible to complement lysis. However, CD59 is not the only potential target and may not even be the best, as evidenced by the requirement for blockade of DAF in order to obtain lysis in our studies. Targeted inhibitory constructs blocking DAF on the cell surface might be particularly useful, either alone or in combination with anti-CD59 constructs. DAF inhibits at the stage of the C3 convertase, and although blocking of DAF alone was not sufficient to render tumour cells susceptible to complement killing in our

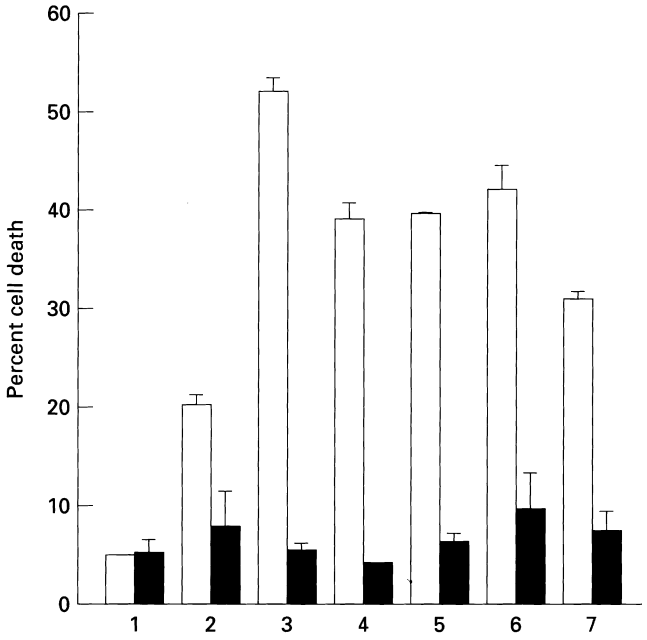

Fig. 6. Specificity of killing using constructs. Raji+3 cells (□) or K562 cells (■) were labelled with the intracellular probe calcein AM. Cells were mixed (5×10^5 cells/ml of each cell type) and sensitized with 10 μg/ml $FabFc_2$ either alone (1), or with anti-decay-accelerating factor (DAF) antibodies (bars 2–7) and other antibodies or constructs (0·1 μg/ml) as follows: 3, anti-CD59 (BRIC229); 4, msAb (MEM43); 5, msAb (YTH53.1); 6, bsAb1 (anti-CD38 × anti-CD59); 7, bsAb2 (anti-CD19 × anti-CD59). Cells were incubated with 1/10 normal human serum (NHS) plus 5 μg/ml $FabFc_2$. Lysis of either Raji+3 or K562 cells was calculated as described in Materials and Methods. Results are means of duplicates ± range.

model, blockade will enhance deposition of C3b on the cell membrane. A coating of C3b on the cell surface, particularly when combined with an array of human Fc, is an excellent stimulus to phagocytosis or to killing of targeted cells through ADCC. This may be an important route for tumour cell clearance *in vivo* following reinfusion of purged bone marrow or therapy *in situ.*

We conclude that antibody constructs designed specifically to increase tumour cell susceptibility to opsonization or killing by homologous complement may be useful both as an adjunct to existing methods of bone marrow purging *ex vivo* [5,30] and for systemic therapy to reduce tumour burden. This approach should be much less prone to problems of toxicity than are conventional immunotoxins, which have numerous deleterious side effects, such as hepatotoxicity and vascular leak syndrome, due to non-specific binding of toxin molecules to bystander cells [31–33]. However, the design of such constructs will require the careful selection of components in order to maximize specificity of delivery and complement-activating capacity, and minimize antigenicity and clearance.

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