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Comparative efficiencies of bispecific F(ab′γ)$_2$ and chimeric mouse/human IgG antibodies in recruiting cellular effectors for cytotoxicity via Fcγ receptors*

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Summary. The three forms of Fcγ receptor carried by monocytes (FcγRI, II) and natural killer (NK) cells (FcγRIII) are all capable of mediating cell lysis. Here we compare the use of F(ab′γ)$_2$ bispecific antibodies, specifically targetting individual FcγR, and chimeric IgG mouse/human antibodies which are capable of targetting all FcγR, for their ability to mediate target cell destruction. The derivatives are prepared by linking hinge sulphydryl residues via tandem thioether bonds, using a bismaleimide crosslinker: Fab′ from an anti-FcγR mAb linked to Fab′ from a common anti-target mAb (BsAb), or Fab′ from the common anti-target mouse antibody linked to human Fcγ (FabFc or bisFabFc). All the derivatives targetting chick red blood cells gave efficient lysis, although different effector cell donors yielded differences in both the lytic levels achieved and the comparative efficiencies of derivatives. In contrast, significant lysis of the guinea pig lymphoblastic leukaemia, L_2C, regularly resulted only via the anti-FcγRIII BsAb and the chimeric derivatives. These results suggest that the chimeric, Fc-containing derivatives mediate tumour cell lysis principally through FcγRIII on NK cells. This is in contrast to the situation with the chick red blood cells where the chimeric derivatives appear capable of lysing erythrocytes by utilizing either monocytes or NK cells, because significant (≈50%) lysis occurred with effector cell populations magnetically depleted through either FcγRII or FcγRIII. A major difference between these two types of antibody derivative was their ability to function in the presence of high concentrations of normal human Fcγ. The lysis mediated by BsAb reactive with FcγRI or II was unaffected by the presence of human Fcγ at 2.5 mg/ml (a concentration comparable with that yielded by IgG in plasma) whereas the BsAb recognizing FcγRIII and all the Fc-containing derivatives were completely inhibited.

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Introduction

The lytic activity of monocytes, macrophages [24] and large granular lymphocytes (LGL/NK cells [22]) is normally triggered when one or more of their various Fc receptors engage the Fc region of antibody coating target cells, e.g. tumour cells. However, more recently bispecific antibodies (BsAb), with dual specificity for tumour and FcR, have also served to direct cellular lytic function very efficiently [8, 19]. Although the nature of the interaction between the receptor and the BsAb (Fab′-FcR) is likely to be very different from the physiological union of Fc-FcR, these derivatives are still able to cause triggering of the effector cells. Potential advantages of BsAb over conventional Fc-displaying antibodies include an ability to select a single type of FcR, and – if the epitope on the FcR is distinct from its binding site for Fc – an avoidance of blocking by Fc on normal Ig.

Although FcR have been described for all classes of Ig on a wide range of haemopoietic cells, it is the FcγR on myeloid and large granular lymphocytes (LGL)/natural killer (NK) cells that continue to attract most attention for triggering cytolysis. There are three major types of human FcγR: FcγRI, II and, III (CD64, CDw32 and CD16 respectively), which differ in molecular mass, cellular distribution, and binding affinity for different subclasses of IgG [31]. FcγRI (72 kDa) is constitutively expressed on human monocytes/macrophages and is inducible on neutrophils after incubation with interferon γ (IFNγ) [3]. This receptor is capable of binding monomeric human IgG1, IgG3 and mouse IgG2a with an affinity constant between 10^8 M^{-1} and 10^9 M^{-1} [32]. FcγRII (40 kDa) is a low-affinity receptor that binds IgG-coated particles or IgG complexes [4] and is expressed on almost all circulating blood cells in-

cluding, monocytes/macrophages, neutrophils, eosinophils, B cells and platelets. As this FcγR has a relatively low affinity for IgG, it was initially thought to be involved solely in the clearance of immune complexes. However more recently it has been shown that FcγRII is capable of delivering a lytic signal in both myeloid and granulocytic cells [17]. FcγRIII (55–70 kDa), which also binds IgG with relatively low affinity, 5×10^5 M^{-1} [28], is the most abundant FcγR on circulating leucocytes, being well presented on neutrophils, LGL/NK cells and macrophages, but not on circulating monocytes. Two allelic forms of FcγRIII have been identified that differ in the mode of membrane linkage [23]. Macrophages, in vitro cultured monocytes and LGL/NK cells all carry the transmembrane-linked form of the receptor, while neutrophils possess a shortened form tethered to the cell surface through a phosphatidylinositol-glycan link. The type of membrane-anchoring appears to play a critical role in determining whether the cell type bearing the receptor is able to mediate target cell lysis through this receptor, as only monocytes and NK/LGL cells, and not neutrophils, are able to trigger significant levels of tumor lysis [8].

Characterising the functions of the different FcR became possible with the production of monoclonal antibodies (mAb) specifically recognising individual receptor types (reviewed in [1]). The role of distinct FcR on various types of effector cells has been clearly seen in reverse-cytotoxicity assays using ^{51}Cr-labelled hybridoma cells bearing surface antibody specific for individual Fc receptors [11]. Fanger and colleagues have shown that hybridoma cells bearing anti-FcγRI or FcγRII were lysed by monocytes, whereas hybridoma cells carrying anti-FcγRIII antibody were lysed by NK/LGL cells. This reflects the cellular distributions of the three forms of FcγR. The use of a wide range of hybridoma cells recognizing other myeloid surface antigens, such as CD14 and CD18, in the reverse-cytotoxicity assays has failed to identify any other myeloid molecule capable of triggering cytolysis [8]. These results strongly support the strategy of harnessing host cellular effectors through their FcγR for the destruction of tumor cells.

Our current work has followed two related strategies in harnessing cytotoxic effector cells via their FcγR. First, two chimeric mouse/human antibody derivatives, FabFc and bisFabFc, have been prepared. These reagents consist of Fab′ from murine mAb chemically linked to human Fcγ via hinge-region sulphydryl groups, as described previously [29]. The FabFc derivative contains one Fab′ and one Fcγ moiety, whereas bisFabFc is a dimer composed of two FabFc molecules. In the second approach, bispecific F(ab′)$_2$ antibodies have been prepared, each containing Fab′ from an appropriate anti-(target cell) antibody chemically linked to Fab′ from a mAb directed against one of the three anti-FcγR antibodies [10]. The efficiency with which these two types of derivative can mediate lysis of chick red blood cells (CRBC) and L_2C tumour cells through various FcγR-expressing human effectors has been investigated, using a large panel of donors, in an effort to determine (a) the derivative with the greatest therapeutic potential and (b) the cell type and individual FcγR best able to mediate cell lysis.

Materials and methods

Cell lines. Hybridoma cell lines were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco Ltd), supplemented with 100 U/ml penicillin (Glaxo, Research Triangle Park, NC, USA), 100 μg/ml streptomycin (Evans), 50 U/ml amphotericin B (Fungizone, Squibb & Sons), 200 mM fresh L-glutamine (Gibco, Middlesex, UK), 100 mM pyruvate and 10% Myoclone plus fetal calf serum (FCS, Gibco). The mAb $E_{11}C_{12}$ [30] and RJD 2A10 [7] directed respectively against CRBC and the L_2C tumor line were raised in this laboratory. The mAb used in this investigation are listed in Table 1. All were of the IgG1 subclass.

Immunoglobulin and Ig fragments. mAb were grown as ascitic fluid in (BALB/c × CBA) F1 mice and purified by ammonium sulphate precipitation and ion-exchange chromatography as described by Elliott et al. [7]. Polyclonal antisera against CRBC and L_2C tumour cells were raised in rabbits using a conventional immunisation schedule [7]: a primary s.c. injection of cells (approx. 10^9) emulsified in complete and incomplete Freund's adjuvant (Difco, Detroit, Mich.) on days 0 and 21, respectively and i.p. in phosphate-buffered saline (PBS) on day 28. The animals were exsanguinated and sera were prepared by allowing the blood to clot overnight at 4° C followed by centrifugation (1800 *g* for 30 min). The IgG fraction was obtained using ammonium sulphate precipitation and ion-exchange chromatography.

F(ab′γ)$_2$ fragments were prepared by pepsin digestion at pH 4.1 according to the method of Lamoyi and Nisonoff [21]. IgG and F(ab′γ)$_2$ were conjugated to fluorescein isothiocyanate (FITC) isomer 1 (BDH, Poole, UK) for immunofluorescence analysis as described by Holborow and Johnson [16].

Fc fragments from normal human IgG were prepared by the method of Stevenson et al. [29], using limited proteolysis with "thio-activated" papain.

Immunofluorescence. The binding of FITC-F(ab′)$_2$ antibody fragments and whole antibody was performed by direct and indirect immunofluorescence, respectively [10]. A FITC-labelled sheep anti-(mouse IgG) was used as the detecting antibody for the indirect analysis. Dual-colour analysis was conducted using directly labelled phycoerythrin-conjugated antibodies as the second reagent. All samples were analysed on a FACScan (Becton Dickinson, Mountain View, Calif., USA).

Preparation of F(ab′γ)$_2$ bispecific antibodies. These derivatives were prepared as described by Glennie et al. [10], by coupling Fab′γ from two different mAb using the chemical linker *o*-phenylenedimaleimide (*o*PDM; Sigma Chemicals Ltd). In all BsAb preparations the mAb, $E_{11}C_{12}$ or RJD 2A10, were used as the target-specific arm for binding to CRBC or L_2C tumour cells respectively. To ensure the removal of any contaminating Fc-containing products, the bispecific derivatives were passed through a column of Sepharose-4B beads (Pharmacia LKB Biotechnology, Uppsala, Sweden) coated with sheep anti-(mouse Fcγ) [30].

Preparation of FabFc and bisFabFc derivatives. FabFc and bisFabFc derivatives were prepared according to the method of Stevenson et al. [29] with modifications. Human Fc, prepared by thio-activated papain digestion of basic human IgG, was reduced with 0.1 volume dithiothreitol (BDH; 66 mM in 10 mM Na_2EDTA) at 37° C for 15 min. The reduced material was then passed through a Sephadex G-25F column (Pharmacia), equilibrated in 0.5 M sodium acetate (NaOAc), 5 mM Na_2EDTA, pH 5.3 and collected on ice, under N_2.

Mouse Fab′γ was obtained from the pepsin digestion of mAb $E_{11}C_{12}$ or RJD 2A10, followed by reduction with 0.2 vol dithiothreitol (6 mM in 10 mM Na_2EDTA) at 37° C for 15 min; 0.3 volume 2,2′-dipyridyldisulphide (Aldrich; 8 mM in 15% dimethylformamide/85% 120 mM acetic acid) was added and the mixture was incubated at 37° C for 15 min before being transferred to an ice bath. Dithiothreitol (6 mM in 10 mM Na_2EDTA), 0.54 volume, was added and the mixture was kept on ice for 30 min before being applied to a Sephadex G-25F column, equilibrated in 20 mM NaOAc, 2 mM Na_2EDTA, pH 5.3. A 0.25 volume of *N,N′-o*-phenylenedimaleimide (10 mM in dimethylformamide) was added to the

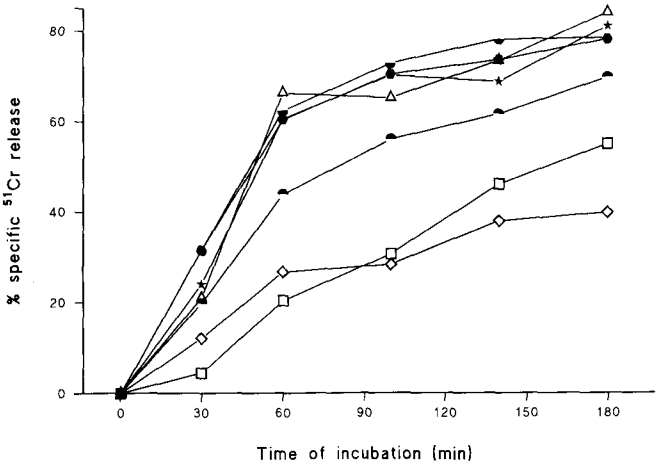


Fig. 1. Time course for lysis of CRBC. All derivatives were used at a final concentration of 5 μg/ml with an effector: target ratio of 10 : 1 in ^{51}Cr-release assays that were harvested after various periods of incubation at 37° C. □, 10.1 × $E_{11}C_{12}$; △, AT10 × $E_{11}C_{12}$; ◇, 3G8 × $E_{11}C_{12}$; ✶, $E_{11}C_{12}$ FabFc; ●, $E_{11}C_{12}$ bisFabFc; ◓, $E_{11}C_{12}$ IgG; ▬, rabbit polyclonal anti-(chick red blood cells, CRBC). Although the line is included, the data points for $E_{11}C_{12}$ bisFabFc are obscured by those for $E_{11}C_{12}$ FabFc or the rabbit polyclonal anti-CRBC. The results are representative of those obtained from three donors

harvested Fab′ material and the solution kept at 5° C for 30 min. Acetic acid (120 mM), 0.15 volume, was added to the mixture, which was then applied to a phospho-Ultrogel column (IBF biotechnics, France), equilibrated in 90% 10 mM Na_2EDTA/10% dimethylformamide. The required $Fab_{(mal)}$ was reverse-eluted with 0.5 M NaOAc, 5 mM Na_2EDTA, pH 5.3, and immediately mixed with the reduced Fc from above. The solution was incubated at room temperature for 2 h. FabFc was separated from material of higher molecular mass and surplus Fc by recycling chromatography on Sephacryl S200HR (Pharmacia) equilibrated in 0.5 M NaOAc, 5 mM Na_2EDTA, pH 5.8 at 5° C. This product was transferred to a protein-A – Sepharose column, equilibrated in 95% 0.5 M NaOAc, 5 mM Na_2EDTA pH 5.3/5% dimethylformamide. 2,2′-dipyridyldisulphide (0.1 mM in column buffer) was passed through the column for 30 min. FabFc was then eluted with 0.1 M glycine/HCl 10 mM Na_2EDTA pH 3.0 and dialysed into PBS.

To synthesize bisFabFc, two preparations of FabFc, of the same or different antibody specificities, were isolated on separate protein A columns. Both FabFc species had their unlinked cysteine (present as 2-pyridyldisulphide) selectively reduced by passage of dithiothreitol (0.7 mM in 0.5 M NaOAc, 5 mM Na_2EDTA pH 5.3) through the columns at 5° C for 30 min. One species of FabFc was then maleimided by passage of *N,N′*-bis(3-maleimidopropionyl)-2-hydroxy-1,3-propanediamine (BMP 8 mM in 87.5% 20 mM NaOAc, 2 mM Na_2EDTA pH 5.3/12.5% dimethylformamide), through the column at 5° C for 30 min. The FabFc fragments were then eluted together by passing 0.1 M glycine/HCl, 10 mM Na_2EDTA pH 3.0 through both columns. The pH was adjusted to 5.3 with NaOAc and the solution was incubated at 20° C for 16 h. Purification of the bisFabFc was again achieved by chromatography on Sephacryl S-200HR.

Effector cell preparation. A monocyte-enriched population of peripheral blood mononuclear leucocytes (PBML) was used as effector cells throughout this study. The method of enrichment was adapted from the protocol reported by Vadas et al. [33]. Briefly, fresh venous blood was taken from healthy volunteers, into preservative-free heparin, and dextran-sedimented with a 6% (w/v) dextran T500 (Pharmacia) solution in 0.9% NaCl (w/v) (1 vol. dextran to 10 vol. blood) for 30 min at 37° C. The upper "leucocyte-rich" fraction was removed, washed twice then resuspended in supplemented DMEM, minus amphotericin B, plus 10% heat-inactivated fetal calf serum and non-essential amino-acids (Gibco), hereafter known as complete media, at $(0.5-2) \times 10^7$/ml. Fractions (2 ml) of the leucocyte-rich population were layered over 3-ml aliquots of an 18% (w/v) metrizamide solution (Nyegaard, Norway) made up in PBS containing 0.1% gelatin (w/v), and spun at 1200 *g* for 30 min. The cells were harvested from the medium/metrizamide interface, washed twice in complete medium and used immediately in cytotoxicity assays. Typically, this population of cells contained 50% – 60% monocytes, 40% – 50% small lymphocytes and 5% – 15% large granular lymphocytes.

Cytotoxicity assays: ADCC and RCC. The protocol used for the cytotoxicity assays was based on that previously described [18]. A pellet of CRBC (approx. 2.5×10^8 cells) or 0.5 ml L_2C cells at 1×10^8/ml in DMEM was labelled by incubation at 37° C with 100 μCi of sodium [^{51}Cr]chromate for 45 min. Free chromium was then removed by four washes in DMEM and the cells were resuspended at 2×10^5/ml in complete DMEM. The assay was performed in U-bottomed 96-well plates (Nunc InterMed, Denmark). ^{51}Cr-labelled target cells (50 μl), were incubated with antibody derivatives or control IgG (50 μl) at various concentrations, in complete DMEM, for 15 min at 4° C before the addition of effector cells (100 μl, 1×10^6/ml) or media, as a control. A final effector: target ratio of 10 : 1 was used as standard. Effector and target cells were brought into contact by centrifugation at 205 *g* for 5 min before incubation for 3 h (CRBC) or 6 h (L_2C) at 37° C in an atmosphere containing 5% CO_2. For CRBC incubation times up to 6 h were used but as these experiments gave higher non-specific ^{51}Cr release with no significant increase in specific lysis, 3-h incubations were adopted as standard. Finally the plates were centrifuged at 432 *g* for 5 min to pellet the cells and 100 μl supernatant was harvested from each well and counted on a gamma scintillation counter (Rackgamma II, LKB). All dilution points were performed in triplicate and mean values determined. The maximum lysis was determined by the addition of 150 μl 1% Nonidet P40 (BDH, Poole, UK) to 50-μl aliquots of target cells. The percentage specific lysis, was calculated from the ^{51}Cr release (cpm) by the following formula:

[(test sample release – control sample release)/(maximum release – control sample release)] × 100.

For blocking studies, additional antibodies were added with the test derivative at appropriate final concentrations, with the total volume maintained at 50 μl.

Immunomagnetic bead cell separation. Fractionation of the effector cell population was performed by negative depletion using immunomagnetic beads coated with sheep anti-(mouse IgG) (Dynabeads M-450, Dynal UK Ltd). The effector cells $[(1-5) \times 10^7$/ml] were incubated with monoclonal IgG of the desired specificity at 100 μg/ml for 30 min on ice, after which the cells were washed once in DMEM and then resuspended in 200 μl medium. Dynabeads M-450 coated with sheep anti-(mouse IgG) were then added at a 40 : 1 ratio of beads to stained cells, in a minimal volume, and incubated on ice for 30 min. The cell/bead slurry was gently agitated every 10 min. Beads and bound cells were collected by placing the tube containing the mixture against a magnetic bar for 2 min after which the medium containing the depleted cell population was removed by pipette. Viability of the cells after this treatment was above 95%. The efficiency of depletion was assayed by flow cytometry.

Results

Cytolytic activity of BsAb and Fc-containing derivatives

The cytolytic activity of monocyte-enriched effector cells from normal donors was investigated using a panel of BsAb specific for FcγRI, FcγRII and FcγRIII, and two chimeric, FabFc and bisFabFc, antibodies containing mouse antibody Fab′ linked to human Fcγ. The target cells for this investigation were either ^{51}Cr-labelled CRBC or L_2C tumour cells. Effector cells from a range of healthy donors were used to investigate the efficiency of lysis

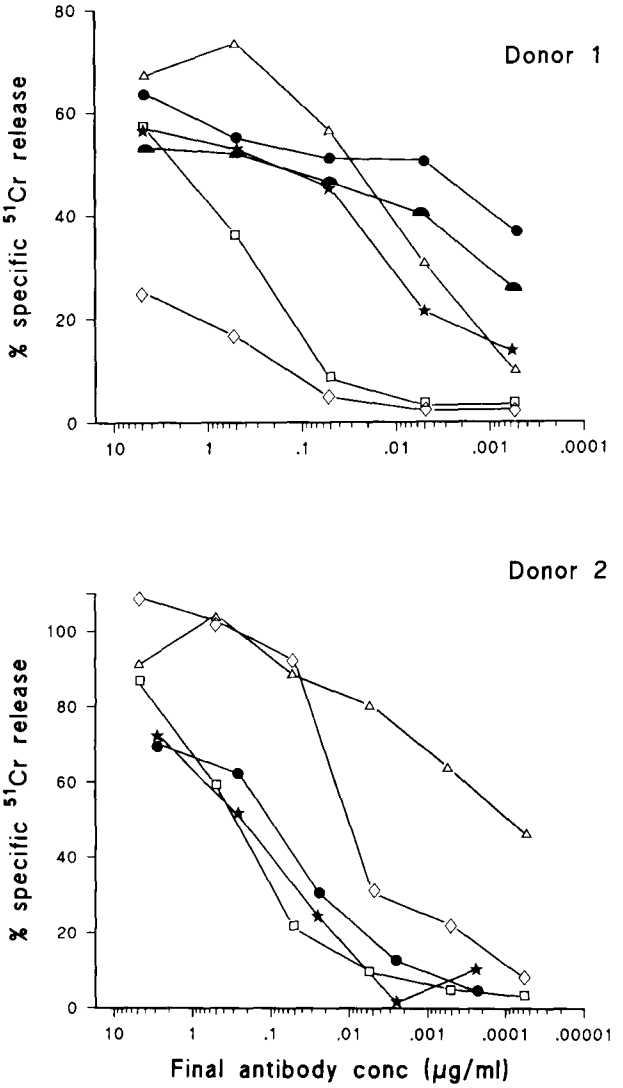


Fig. 2. Lysis of CRBC mediated by F(ab′γ)$_2$ bispecific and mouse/human chimeric antibodies. Bispecific derivatives targetting each FcγR were compared with chimeric antibodies for the ability to mediate lysis. All derivatives were used at a final concentration of 5 μg/ml with an effector: target ratio of 10 : 1 in a 3-h ^{51}Cr-release assay. *Donor 1* and *Donor 2* are representative of the two different lysis patterns given by the panel of derivatives. Effector cells from nine independent subjects gave patterns like donor 1 and a further nine subjects gave patterns like donor 2. □, 10.1 × $E_{11}C_{12}$; △, AT10 × $E_{11}C_{12}$; ◇, 3G8 × $E_{11}C_{12}$; ✶, $E_{11}C_{12}$ FabFc; ●, $E_{11}C_{12}$ bisFabFc; ◓, $E_{11}C_{12}$ IgG. Note that no data curve for $E_{11}C_{12}$ IgG is shown for donor 2 but in other experiments this reagent gave lysis similar to $E_{11}C_{12}$ FabFc

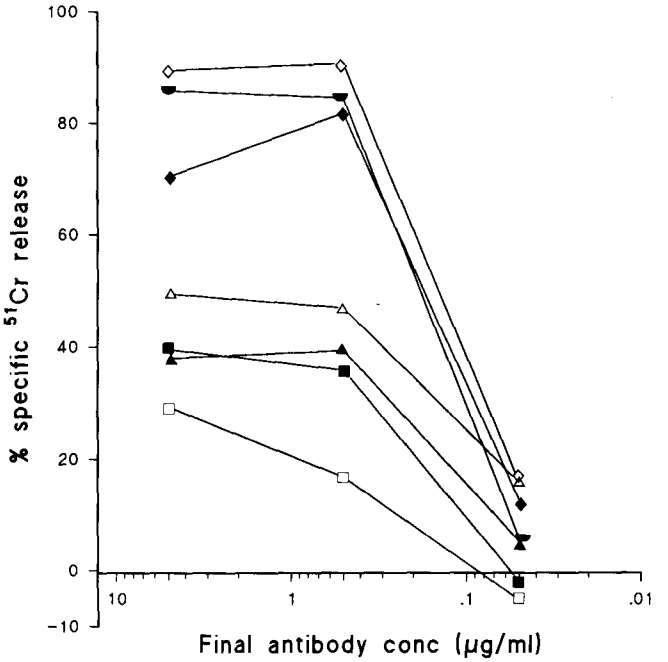


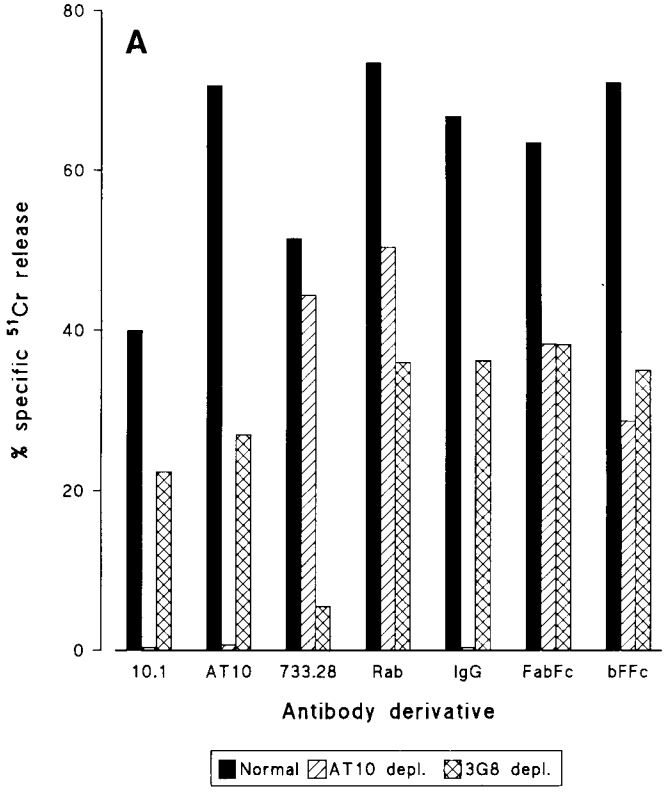
Fig. 3. Lysis of L_2C tumour cells mediated by F(ab′γ)$_2$ bispecific antibodies. Bispecific antibodies were constructed from two different mAb targetting each FcγR and were tested for their ability to lyse L_2C target cells. All derivatives were used at a final concentration of 5 μg/ml with an effector: target ratio of 10 : 1 in a 6-h ^{51}Cr-release assay. The data shown are representative of five donors. □, 10.1 × RJD; ■, 22 × RJD; △, AT10 × RJD; ▲, KB61 × RJD; ◇, 3G8 × RJD; ◆, 733.28 × RJD; ◒, rabbit polyclonal anti-L_2C

mediated by the various derivatives. Results in Fig. 1 show that the time course for lysis of CRBC with each of the derivatives was similar. Maximum lysis was normally achieved within 3 h as shown. However, with certain, more active, effectors near-maximal lysis was reached within 1 h (data not shown).

The cytolytic activity of each derivative was investigated over a wide range of concentrations. Representative results from these assays are shown in Fig. 2 and 3. A number of general factors emerge: first, we found significant donor variation in the maximum levels of lysis achieved; second, the lytic activity of effector cells from one individual, when used on multiple occasions, tended to remain either high or low for any particular derivative; finally, the cytolytic activity of FabFc and bisFabFc derivatives were very similar and equivalent to that of control, rabbit polyclonal IgG.

No derivative, BsAb or Fc-containing, was consistently superior in mediating destruction of the CRBC. In total we investigated effector cells from 22 healthy donors. With nine of these, the anti-FcγRII-specific BsAb, (AT10 × anti-CRBC, KB61 × anti-CRBC), and the Fc-containing derivatives proved most effective, giving lysis profiles similar to those shown by donor 1 in Fig. 2. Effectors from a further nine donors performed in a manner similar to those from donor 2 in Fig. 2, with the anti-FcγRII- and anti-FcγRIII-specific BsAb [3G8 × anti-CRBC, 733.28 × anti-CRBC (data not shown)] proving the superior reagents. Cells from the remaining four donors gave similar levels of lytic activity with all derivatives.

In contrast to the CRBC data, experiments with the L_2C targets, although still showing donor variation in the levels of lysis achieved, gave a clearer hierarchy of performance for the different derivatives (Fig. 3). The anti-FcγRIII × anti-Id and Fc-containing derivatives were the most active reagents and clearly superior to the BsAb targetting FcγRI and FcγRII. The performance of each type of BsAb was confirmed using "matched" pairs of BsAb (Fig. 3). Each pair of reagents was constructed with the same anti-Id antibody recognising the L_2C targets, but different anti-FcγR antibodies to bind to each type of FcγR. The results in Fig. 3 show that each member of these



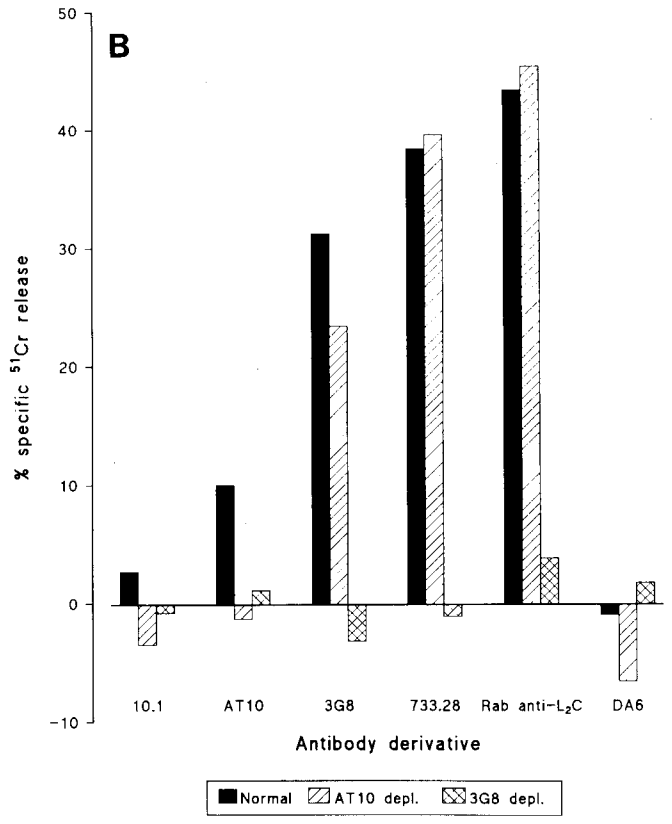


Fig. 4 A, B. Lysis of CRBC and L_2C cells mediated by immunomagnetically depleted effector cell populations. Effector cells were depleted with either mAb AT10 (anti-FcγRII) or 3G8 (anti-FcγRIII) and the residual populations were then used in standard 3-h (CRBC, **A**) or 6-h (L_2C, **B**) ^{51}Cr-release assays. All antibody derivatives were used at a final concentration of 5 μg/ml with an effector: target ratio of 10 : 1. *Axis labels:* 10.1, AT10, 733.28, 3G8 and DA6 are all bispecific antibodies with the other arm being $E_{11}C_{12}$ (anti-CRBC) or RJD (anti-L_2C) respectively. *Rab*, rabbit polyclonal anti-CRBC; IgG, $E_{11}C_{12}$ IgG1; FabFc, $E_{11}C_{12}$ FabFc; bFFc, $E_{11}C_{12}$ bisFabFc. Results are representative of those from six donors

matched pairs of reagents had very similar cytolytic activity, showing that the activity of BsAb was dictated largely by the FcγR being recognised.

Cytotoxicity after effector cell depletion

Depletion studies using immunomagnetic beads were conducted to investigate the nature of the effectors responsible for lysis with the various derivatives. Effector cell populations were depleted with either anti-FcγRI (10.1), anti-FcγRII (AT10) or anti-FcγRIII (3G8) antibodies, and residual cells were used in standard cytotoxicity assays. Flow cytometry analysis showed that depletion through FcγRII or FcγRIII was very efficient and removed almost all detectable monocytes (>95%, confirmed by staining with anti-FcγRI or anti-CD14 mAb) or NK cells (>90%, confirmed by staining with anti-CD56 mAb) respectively. However, for unexplained reasons, depletion with anti-FcγRI was unsuccessful and left up to 40% of the monocytic cells in the residual population. For this reason anti-FcγRI-depleted cells were not used in cytolytic assays.

We often found that the manoeuvres needed to achieve depletion tended to reduce the cytotoxic activity of effector cells regardless of whether specific effector cells had been removed. Representative results using depleted populations against CRBC and L_2C are shown in Fig. 4. Depleting with anti-FcγRII (AT10) removes all killing of CRBC via FcγRI (10.1 × anti-CRBC, 22 × anti-CRBC) and FcγRII (AT10 × anti-CRBC, KB61 × anti-CRBC), but only slightly decreased cytotoxicity mediated via FcγRIII-specific BsAb or the human Fc-containing derivatives. These results indicate that the FcγRII depletion removes, among other cells, the FcγRI$^+$ monocytes thus preventing killing via FcγRI and FcγRII. However, this does not interfere with cytolysis mediated by FcγRIII$^+$ effectors (NK cells), which remain to be recruited by anti-FcγRIII-specific BsAb (3G8 × anti-CRBC, 733.28 × anti-CRBC) or human-Fc-bearing reagents. The IgG1 anti-CRBC monoclonal antibody, $E_{11}C_{12}$, behaved differently from other Fc-containing derivatives in that it mediated no lysis after depletion via anti-FcγRII, indicating that this antibody was inactive with the FcγRIII$^+$ effectors – presumably because of the failure of mouse IgG1 to bind to FcγRIII [1]. Similar results were obtained with the L_2C targets. In the example shown (Fig. 4B), removal of the FcγRII$^+$ cells had no effect on the cytolytic activity of anti-FcγRIII-specific BsAb or a rabbit anti-L_2C IgG antibody. The effect of this depletion on killing of L_2C via FcγRI and FcγRII was more

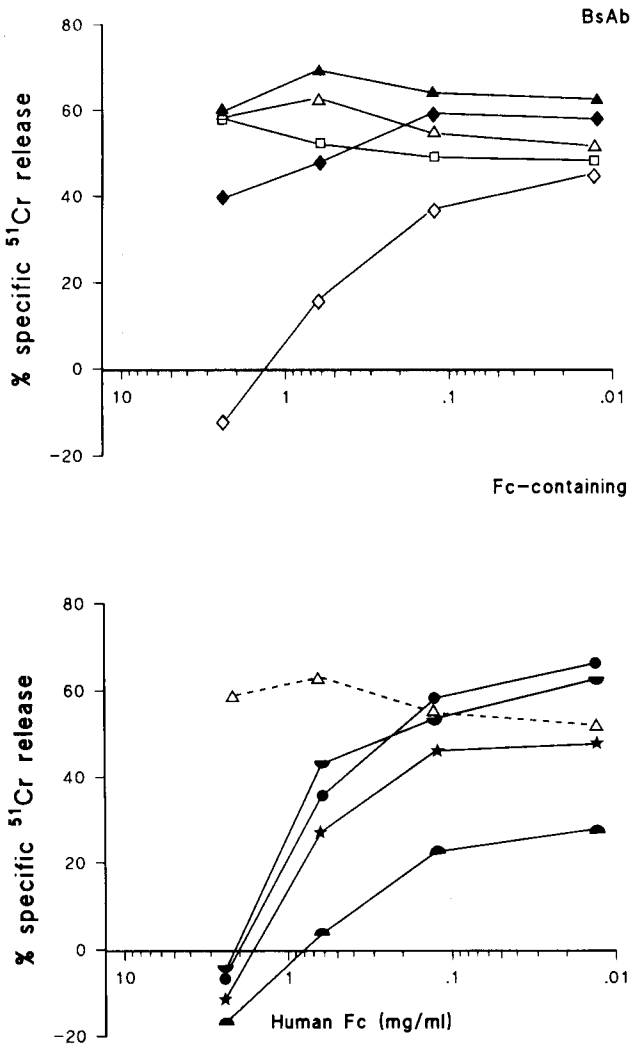


Fig. 5. Blocking of lysis by the presence of human Fcγ. Various concentrations of human Fc were incubated with the test derivatives that were present at 5 μg/ml. The level of inhibition of lysis was measured in a standard 3-h ^{51}Cr-release assay, with an effector: target ratio of 10: 1. Results shown are representative for three donors. □, 10.1 × $E_{11}C_{12}$; △, AT10 × $E_{11}C_{12}$; ▲, KB61 × $E_{11}C_{12}$; ◇, 3G8 × $E_{11}C_{12}$; ◆, 733.28 × $E_{11}C_{12}$; ✶, $E_{11}C_{12}$ FabFc; ●, $E_{11}C_{12}$ bisFabFc; ◓, $E_{11}C_{12}$ IgG; ◒, rabbit polyclonal anti-CRBC

difficult to assess, because of the low level of cytotoxicity achieved via these receptors.

Conversely, depletion of FcγRIII$^+$ effectors with the mAb 3G8 totally abolished all killing mediated via FcγRIII using either of the matched pair of BsAb (3G8 × anti-target, 733.28 × anti-target) against CRBC or L_2C targets. Removal of these cells also prevented lysis by the rabbit IgG anti-L_2C reagent showing that the FcγRIII$^+$ cells in PBML are the major effectors in lysing L_2C tumour cells. However, in the case of the CRBC targets (Fig. 4 A), the Fc-containing derivatives showed significant lytic activity with these FcγRIII$^-$ effectors, suggesting that CRBC are more susceptible than L_2C to lysis mediated via FcγRI and FcγRII. Unfortunately, owing to the variable loss in lytic activity that often accompanies the preparation of effectors by antibody-depletion, it is difficult accurately to quantify the relative contributions of the different FcγR to killing of CRBC by Fc-containing derivatives.

Table 1. Antibodies used in this study

mAb	Specificity	Source
10.1	FcγRI	N. Hogg (ICRF, London) [6]
22	FcγRI	M. Fanger (Dartmouth, USA) [2]
KB61	FcγRII	D. Mason (John Radcliffe Hosp, Oxford) [25]
AT10	FcγRII	Laboratory [12]
3G8	FcγRIII	D. Segal (N. I. H., USA) [9]
733.28	FcγRIII	E. Roosnek (Basel, Switzerland) (unpublished)
DA6.231	MHC class II	K. Guy (MRC, Edinburgh)
WR18	MHC class II	J. Smith (Wessex Region, Southampton)
$E_{11}C_{12}$	CRBC	Laboratory
RJD 2A10	L_2C idiotype	Laboratory
LeuM3	CD14	Becton Dickinson
Leu11	CD16	Becton Dickinson
Leu19	CD56	Becton Dickinson

Attempts to confirm the depletion studies using FcγR-independent markers, LeuM3 (CD14) for monocytes and Leu19 (CD56) for NK cells, were not completely satisfactory. While depletion with anti-CD14 removed all the lytic activity of the anti-FcγRI-specific BsAb and some of that mediated through FcγRII, depletion with anti-CD56 had minimal effect on lysis by any of the derivatives. Flow cytometry analysis indicated incomplete depletion of effectors with these antibodies (data not shown).

Blocking cytolysis with human Fc

In vivo, plasma IgG is present at approximately 12 mg/ml (human IgG1 at 8 mg/ml), so that any therapeutic antibody reagent will need to compete with this barrier of circulating immunoglobulin. In these experiments we have added human Fcγ, prepared principally from human IgG1, up to a final concentration of 2.5 mg/ml to attempt to mimic the effects of circulating IgG (Fig. 5). Results show that all the Fc-containing derivatives and the anti-FcγRIII BsAb (3G8 × anti-CRBC, 733.28 × anti-CRBC), when used at optimal conditions, are completely blocked by the addition of human Fcγ at 2.5 mg/ml (under these conditions the Fcγ is present at a 500-fold excess by weight). The results suggest that mAb 733.28 recognises a spatially close but possibly not identical epitope to that seen by mAb 3G8 and the IgG binding site. In contrast, the anti-FcγRI and anti-FcγRII BsAb are not blocked even at the highest concentration of Fc.

Discussion

The Fcγ receptors have been shown to be key surface molecules in regulating various activities of a wide variety of cell types [20, 32]. The aim of the current work was to assess the relative contribution of individual FcγR in triggering cellular cytotoxicity by monocytes and NK cells using various antibody derivatives. In addition, we have compared the activity of F(ab′γ)$_2$ BsAb, which recruit effectors via a single FcγR with two mouse/human chimeric Fc-containing derivatives, FabFc and bisFabFc, which are expected to function through multiple FcγR.

Initial studies showed that all derivatives, bispecific and chimeric, were capable of recruiting the appropriate FcγR-bearing effectors for lysis of CRBC and L_2C tumour cells. Using a monocyte-enriched preparation of PBML, which contained 50%–60% monocytes and 5%–15% large granular lymphocytes, optimal lysis was achieved in a 3-h ^{51}Cr-release assay with an effector:target ratio of 10:1. Our results were characterised by wide donor variation in the levels of lysis achieved and, in the case of the CRBC targets, no clear hierarchy in the activity levels of the different types of derivative was obtained. Thus, with effector cells from some donors we found evidence of an advantage with the Fc-containing derivatives, FabFc and bisFabFc, while with others it was the BsAb, particularly an anti-FcγRII-specific reagent, which appeared more active. A consistent finding from this part of the work was that, for any particular batch of effector cells from one donor, the Fc-containing reagents, FabFc and bisFabFc, gave very similar levels of activity, presumably because they were utilizing the same type of effector cells. In contrast, the different BsAb, operating through the individual FcγR, differed widely in performance. Also from this work it would appear that the bisFabFc, with the two juxtaposed Fc regions, gains no advantage over the single Fc in FabFc when activating cellular cytotoxicity of CRBC, even though previously it has been shown that this molecule is far more efficient at mediating complement lysis and gives a small benefit in tumour cell lysis [29].

Lysis of the more resistant L_2C tumour cells gave a far clearer picture of differences in performance of the various reagents. Rabbit polyclonal antibody, the chimeric derivatives and BsAb containing anti-FcγRIII antibody all behaved very similarly and were far superior to BsAb operating via anti-FcγRI or anti-FcγRII. The experiments with L_2C targets indicated that we were recruiting at least two types of effector: first, highly active cells, probably NK cells, recruited by Fc-containing derivatives and the anti-FcγRIII-specific BsAb; and second, poorly cytotoxic monocytes bound by BsAb containing anti-FcγRI and anti-FcγRII. In many cases this latter type of reagent, which had been fully effective with CRBC, gave minimal or no lysis of L_2C. The marked difference in performance between a BsAb acting via FcγRIII and those binding FcγRI or FcγRII was confirmed by using matched pairs of reagents, each containing the same anti-(target cell) antibody (RJD 2A10) with different anti-FcγR antibodies. The results showed very clearly that the activity of the different type of BsAb depended on the FcγR being recruited and not some other property of the antibody derivative such as its affinity or the topography of the epitope being recognised.

Studies using immunomagnetically depleted effector populations identified NK cells and monocytes as the two cell types in our monocyte-enriched PBML preparation. In the case of the L_2C targets, depletion of effectors with the anti-FcγRIII mAb (3G8) removed all lytic activity regardless of which type of derivative was being used. Unfortunately, attempts to deplete these same effectors via an LGL/NK surface molecule, CD56, which will not be directly involved in the antibody-recruiting process, were unsuccessful. Thus we cannot be sure that some of the effects thought to result from depletion were merely the consequence of blocking by mAb 3G8. However, regardless of the mechanism of inhibition, our results show, as proposed by Dearman et al. [5], that killing of the L_2C by Fc-containing derivatives was primarily mediated by FcγRIII$^+$ effectors with little or no role for the FcγRI$^+$ and/or FcγRII$^+$ cells, such as monocytes.

Cytolysis of the more sensitive CRBC targets again showed that FcγRIII$^+$ effectors are important, but in this case the FcγRI- and FcγRII-expressing cells were also active. These results confirm the work of Ruegg and Jungi [27] and Van de Winkel et al., [34], who showed, using red blood cells opsonized with rabbit or mouse IgG antibody respectively, that both forms of Fcγ receptor on monocytes are involved in erythrocyte lysis by classical ADCC. Depletion with mAb 3G8 (anti-FcγRIII) totally removed, or blocked, all killing mediated by anti-FcγRIII BsAb, likewise purging with an anti-FcγRII antibody (AT10) prevented lysis mediated by the BsAb containing anti-FcγRI or anti-FcγRII. The removal of all FcγRI killing by depletion with the anti-FcγRII antibody is highly unlikely to be due to blocking of the receptor and this supports the hypothesis, reinforced by flow cytometry, that the beads are physically removing the effector cells. Further depletion studies and dual-colour flow-cytometric analysis with an anti-CD14 mAb (LeuM3), which is reported to be a highly specific marker for monocytes [15], confirmed that the anti-FcγRI- and anti-FcγRII-specific BsAb were triggering the monocyte population.

In contrast, depletion with anti-FcγRII or III failed to remove all the activity of the Fc-containing derivatives, but tended to reduce the level of ^{51}Cr release by about 50%. We conclude from these findings that, for the killing of CRBC at least, although the BsAb can recruit only one type of effector cell: NK (via FcγRIII) or monocyte (via FcγRI and FcγRII), the IgG chimeric derivatives can use both. The results from the two types of target highlight the difficulty in extrapolating from various target systems in which different cytotoxic effector-cell mechanisms may be used.

An important difference between the two classes of derivatives was shown when the cytotoxicity assays were performed in the presence of normal human Fc, attempting to mimic the environment in blood where IgG is present at a concentration of approximately 12 mg/ml (equivalent to 4 mg/ml human Fcγ). The results show clearly that all cytotoxicity mediated by the Fc-containing antibodies, present at optimal concentrations, is completely blocked by the presence of Fc at 2.5 mg/ml, as is that of the anti-FcγRIII BsAb; whereas the cytotoxicity mediated by monocytes using the anti-FcγRI or anti-FcγRII BsAb is unaffected. There are two possible explanations for this non-blockable cytolysis by BsAb on monocytes: first, that the mAb recognizes an epitope outside of the normal Fc-binding site and second, that the mAb binds an epitope near, or in, the binding site but has an affinity constant several orders of magnitude greater than that of the normal Fc-Fcγ receptor interaction and therefore competes successfully with the Fc fragments. The first explanation is probably correct for FcγRI as neither mAb 10.1 or 22 was able to inhibit the binding of IgG to FcγRI$^+$ cells [6, 14] and therefore these derivatives were presumably triggering lysis by targetting FcγRI outside the Fc-ligand binding site.

The second explanation appears true for FcγRII; Fab′ from both mAb AT10 or KB61 significantly inhibited rosette formation between CRBC opsonised with rabbit IgG and FcγRII-positive cells, thus demonstrating that these antibodies recognise a site near, or in, the Fc-binding site [12]. Also, Fab′ from AT10 mAb has an affinity constant (K_a) of 5×10^8 M^{-1} for FcγRII [12], a more than 100-fold increase in affinity over monomeric IgG binding to FcγRII (10^6 M^{-1}) [4]. As the Fc-containing derivatives all function through Fc-Fcγ receptor binding it is not surprising that, in the presence of a vast excess (1000-fold) of irrelevant Fc, these derivatives are ineffective since the probability of the targetting reagent binding to a FcγR is minimal.

Our results show that not all BsAb overcome blocking by Fcγ. The exception was found when NK cells were recruited through FcγRIII. In this case Fc completely blocked BsAb made with 3G8 and partially blocked those made with 733.28. The mAb 3G8 is known to bind near, or at, the binding site for IgG on FcγRIII [9] but, unlike mAb AT10 and KB61 binding to FcγRII, it appears that the excess of human Fc is able to compete successfully with these antibodies. This difference between BsAb made with anti-FcγRIII and anti-FcγRII may be explained simply by affinities of the mAb, or it may reflect some other feature of the FcγRIII, such as a subtle conformational change or surface aggregation on binding IgG, which prevents binding by a mAb.

In conclusion, our results suggest that derivatives relying on recruitment via FcγR will be cytolytically effective against tumour only via FcγRIII. As a corollary the cytotoxic mononuclear cells involved must be of the LGL/NK type. For use in vivo either the chimeric derivative or an anti-FcγRIII BsAb would be the derivative of choice. Greater efficacy might be achieved by activating the effector cells, e.g. LGL cells cultured in IL-2 to produce lymphokine-activated killer cells [13], as these cells have been shown to be beneficial in some cases against autologous tumours [26]. Factors such as tumour susceptibility to complement lysis, only mediated by the chimeric antibodies, will also influence the final choice of antibody derivative. The potential advantage of the BsAb as opposed to the chimeric derivatives is that they can function in the presence of serum concentrations of human Fcγ. Unfortunately, both anti-CD16 mAb used in the current work to prepare BsAb were partially or completely blocked. Our goal now must be the construction of an anti-FcγRIII BsAb, which is completely resistant to blocking for subsequent therapeutic evaluation.

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