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The functional activity of FcγRII and FcγRIII on subsets of human lymphocytes

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SUMMARY

Subsets of human lymphocytes were isolated from peripheral blood using magnetic beads coated with anti-CD4, -CD8, -CD19 or -CD56 antibodies to yield T4, T8, B and natural killer (NK) cell suspensions with greater than 95% purity. The functional activity of Fcγ receptor II (FcγRII) and Fcγ receptor III (FcγRIII) on these subsets was assessed by measuring rosette formation with red cells sensitized with known levels of either rabbit IgG or human (monoclonal or polyclonal) IgG1 anti-D, IgG3 anti-D or IgG3 anti-c (E-IgG). Lysis of red cells by K cells (mediated by FcγRIII) in antibody-dependent cell-mediated cytotoxicity (ADCC) assays was promoted by polyclonal and some monoclonal antibodies. Using these 'ADCC$^+$' antibodies, minimum red cell sensitization levels required to promote rosette formation with NK cells were 2000 IgG1 or IgG3 molecules/red cell compared to 15,000 IgG1 or 4000 IgG3 molecules/red cell with 'ADCC$^-$' monoclonal antibodies. The greater efficiency of ADCC$^+$ antibodies is consistent with their previously reported ability to bind FcγRIII via CH2 and CH3 domains whereas ADCC$^-$ antibodies bind only via CH3 domains. B cells formed rosettes only at high levels of sensitization: approximately 60,000 IgG1 or 20,000 IgG3 anti-D molecules/cell. These data reflect the low affinity of FcγRII for monomeric human IgG. Although over 90% of NK cells bound anti-CD16, and 70% formed rosettes with red cells sensitized with rabbit IgG (30,000 molecules/cell), only 25% of NK cells formed rosettes with E-IgG3 at 100,000 IgG molecules/cell. Approximately 35% of B cells, 10% of T8 cells but no T4 cells formed rosettes with E-IgG (100,000 IgG3 molecules/cell). With T8, B and NK cells, IgG3 anti-D promoted greater rosette formation than IgG1 anti-D at comparable levels of sensitization. Presumably the longer hinge region of IgG3 enabled it to bridge the gap between negatively charged lymphocytes and red cells more efficiently than IgG1.

INTRODUCTION

The use of monoclonal antibodies against CDw32 and CD16 and analyses of amino acid sequences from cDNA have recently revealed much about the distribution and structural heterogeneity of Fcγ receptor II (FcγRII) and Fcγ receptor III (FcγRIII) on human leucocytes.[1] The biological functions of these FcγR have been investigated using a variety of approaches. Monoclonal antibodies have been used to cross-link FcγR on effector cells, and such studies have shown, for example, that cross-linking of all three classes of FcγR on myeloid cells may trigger superoxide generation.[2–4] Other workers have shown that different FcγR are capable of mediating phagocytosis and ADCC using bifunctional or heteroconjugate antibodies to link leucocytes via their receptors to a variety of target cells.[5] However, studies such as these reveal little about the nature of interactions between receptors and their 'natural' ligand, the Fc region of human IgG. Classical methods such as rosette formation have highlighted the role of the high-affinity FcγRI on monocytes in promoting the recognition and destruction of red cells sensitized with anti-D.[6] However, similar studies on FcγRII and FcγRIII on lymphocytes have been hampered by the relatively low affinity of these receptors for human IgG, and the difficulty in obtaining pure lymphocyte subsets.

Previous studies[7–9] showed that approximately 10% of non-adherent mononuclear cells formed rosettes with human red cells sensitized with 20,000 IgG3 molecules/cell. Using sera which contained only detectable IgG1 or IgG3 anti-D, IgG3 was shown to be the more efficient subclass at promoting rosette formation. In the current study, these observations have been extended by using -D- phenotype red cells (with 150,000 D sites/cell) sensitized with human monoclonal antibodies, and a sensitive solid-phase rosette assay[10] to compare the ability of subsets of lymphocytes to form rosettes. The subsets were positively selected using immunomagnetic separation and defined by their reactivity with monoclonal antibodies: CD19$^+$ B cells, CD4$^+$ and CD8$^+$ T cells, and CD3$^-$, CD56$^+$, CD16$^+$ natural killer (NK) cells. A further objective was to compare the functional activity of monoclonal and polyclonal anti-D.

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Earlier studies[9] showed that lymphocytes bound relatively less well to red cells sensitized with some monoclonal anti-D, an observation which merited further investigation in view of the possibility of replacing plasma-derived polyclonal anti-D with a monoclonal preparation for prophylaxis against haemolytic disease of the newborn.[11]

MATERIALS AND METHODS

Antibodies and preparation of sensitized red cells

Human monoclonal antibodies were used as culture supernatants[12,13] comprising IgG1 anti-D (AB5, JAC10, 1A11 and R1D7), IgG3 anti-D (1A3−3 and FEF7) and IgG3 anti-c (BB7.D4, a gift from Professor P. Rouger, Institute National de Transfusion Sanguine, Paris, France). Sera containing polyclonal IgG1 (Mysl and Mir) or IgG3 (Krzep) anti-D have been described previously.[7] IgG2 anti-D[14] was purified from an unusual serum (B. M. Kumpel, unpublished observations). Anti-D immunoglobulin for Rh prophylaxis (containing predominantly IgG1 anti-D) was obtained from Bio-Products Laboratory (Elstree, U.K.). Chicken, ox (Serotec, Oxford, U.K.) or human (phenotype: R2R2 or -D-) red cells (5% v/v in Dulbecco's modified Eagle's medium containing 10% v/v foetal calf serum; DMEM/FCS) were sensitized with equal volumes of appropriate dilutions of rabbit IgG anti-red cell (Cappel, Cambridge, U.K.), human anti-D or anti-c. Levels of red cell-bound immunoglobulin were determined by ELISA.[15]

Antibody-coated magnetic beads

All procedures were performed at 4°. Anti-CD56-coated beads were prepared by incubating 0·05 ml (1·5 mg) Dynabeads™ (M450 beads; Dynal, Wirral, U.K.) precoated with goat anti-mouse IgG with 0·03 ml (1·5 μg) anti-Leu-19 (Becton Dickinson, Oxford, U.K.) for 12 hr. Beads were then washed four times in phosphate-buffered saline containing 1% v/v foetal calf serum (PBS/FCS; Imperial Laboratories, Andover, U.K.) using a magnetic separator (Dynal) and concentrated to a pellet. Anti-CD4, -CD8 and -CD19-coated beads were purchased from Dynal. Twenty microlitres of each were washed and concentrated to a pellet as described above.

Preparation of lymphocyte subsets

Twenty millilitres of blood from a single donor or from a pool of six individuals was anti-coagulated in disodium ethylenediaminetetraacetic acid. Mononuclear cells were isolated by density gradient centrifugation (Histopaque; Sigma, Poole, Dorset, U.K.), washed thrice with PBS/FCS, resuspended in DMEM/FCS and depleted of monocytes by adherence to plastic for 2 hr at 37°. Non-adherent cells were washed, resuspended in 5 ml PBS/FCS and T4 and T8 cells isolated by adding 2·5 ml aliquots to beads precoated with anti-CD4 or anti-CD8. Cells and beads were gently mixed for 45 min at 4°, rosetted cells were removed using a magnetic separator (Dynal) and the supernatants were retained and mixed. Beads with adherent lymphocytes were washed twice with ice-cold PBS/FCS and then resuspended in approximately 0·2 ml PBS/FCS. Ten microlitres DETACHaBEAD™ (Dynal) was added to each tube, and incubated at room temperature for 45 min during which time the beads were encouraged to detach from the cells by occasional vigorous mixing. Beads and detached cells were separated, and the cells washed and resuspended in PBS.

B cells were prepared by adding the combined supernatants to beads coated with anti-CD19. Rosette formation and bead detachment were achieved as described above, and the cells resuspended in PBS.

NK cells were similarly isolated using beads coated with anti-CD56 (adapted from Naume *et al.*).[16] After rosette formation and bead detachment, cells were resuspended in 0·5 ml PBS/FCS. Contaminating T cells, monocytes and B cells were removed by incubating cells with 10 μl of anti-CD3, -CD14, and -CD19 (Serotec) for 30 min at room temperature. Cells were then washed, depleted of sensitized cells as described above and resuspended in PBS.

The purity of lymphocyte subsets and FcγR expression was assessed by flow cytometry (Becton Dickinson FACStar) using monoclonal antibodies against CD3, CD4, CD14, CD19 (Serotec), CD8, and CD3, 16, 56 (Simultest™ reagent) (Becton Dickinson) and CDw32 (clone 2E1, Serotec).

Rosette formation

Lymphocytes were suspended in PBS at 1×10^5 cells/ml, and 0·2 ml aliquots added to 48-well tissue culture plates which had been previously coated with 0·1 mg/ml poly-L-lysine (MW = 250,000) for 2 hr.[10] Plates were then centrifuged at 200 *g* for 10 min, the PBS was decanted and the wells blocked with DMEM containing 15% FCS for 90 min at room temperature. The DMEM was decanted and replaced with 0·2 ml sensitized or control red cells (1% v/v in DMEM/FCS). Plates were centrifuged at 200 *g* for 5 min then incubated at 4° overnight. Cells were fixed by addition of 0·1 ml glutaraldehyde (4% v/v in PBS) for 10 min and then non-rosetted red cells were rinsed away with PBS. Staining was achieved using acridine orange (1 mg/ml), and the percentage of cells with one or more attached red cell was estimated using an inverted fluorescent microscope. Rosette formation of 3% or greater was considered positive.

Antibody-dependent K-cell-mediated cytotoxicity

Lysis of ^{51}Chromium-labelled papainized R2R2 red cells by non-adherent lymphocytes in the presence of AB serum and fluid-phase antibodies was performed as described previously.[12]

Measurement of zeta potential

The zeta potential of red cells was determined by measuring electrophoretic mobility in a Zetasizer IIc (Malvern Instruments, Malvern, U.K.). Measurements were made using cells suspended in PBS (pH 7·3) at 25° in a field strength of 32 V/cm. Results were expressed as the mean velocity of cells.

RESULTS

Purity and phenotypic analysis of lymphocyte subsets

The relatively low number of cells required for the solid-phase rosette assays permitted the isolation of lymphocyte subsets by 'positive selection' using antibody-coated magnetic beads. Cell detachment from the beads was achieved with greater than 80% efficiency by incubation with goat anti-mouse Fab antibody (DETACHaBEAD™; Dynal). Since isolation procedures (with the exception of incubation with DETACHaBEAD) were performed at 4°, activation of cell populations was unlikely. Further, the binding of anti-CD16 and anti-CDw32 to lymphocyte subsets before and after isolation was comparable (Table

1), suggesting that FcγR expression was unaffected. Cytometric analyses revealed the lymphocyte subpopulations to be consistently over 95% pure with less than 2% monocytes. However, despite the depletion of monocytes by adherence, NK cell preparations required further depletion steps using magnetic beads to achieve these levels of purity. NK cells were also depleted of $CD3^+$ cells to remove a subset of $CD56^+/CD3^+$ cells;[17] less than 1% NK cells stained with anti-CD3 after depletion.

K-cell-mediated red cell lysis

Table 2 shows that antisera containing polyclonal IgG1 or IgG3 anti-D, and culture supernatants containing monoclonal IgG1 anti-D (1A11 and R1D7) and IgG3 anti-c (BB7.D4) promoted over 85% red cell (R2R2) lysis when tested undiluted (designated $ADCC^+$ antibodies). Monoclonal IgG1 anti-D (AB5 and JAC10), IgG3 anti-D(1A3-3 and FEF7), and polyclonal IgG2 anti-D promoted less than 30% lysis (designated $ADCC^-$ antibodies).

Table 1. CD16 and CDw32 expression on NK, B, T4 and T8 cells before and after isolation. The data show the mean peak channel fluorescence and (in parentheses) the percentage of each subset expressing either CD16 or CDw32. Results are from a single typical experiment

	Binding of monoclonal anti-FcγR antibodies. Mean peak channel fluorescence (% positive cells)	
	Anti-CD16	Anti-CDw32
NK cells, pre-isolation	256 (ND)	NA
NK cells, post-isolation	272 (95·7)	NA
B cells, pre-isolation	NA	176 (91·0)
B cells, post-isolation	NA	167 (78·9)
$CD4^+$ cells, pre-isolation	ND (< 1%)	NA
$CD4^+$ cells, post-isolation	ND (< 1%)	NA
$CD8^+$ cells, pre-isolation	36 (17·4)	NA
$CD8^+$ cells, post-isolation	27 (15·6)	NA

ND, not determined; NA, not applicable.

Rosette formation with sensitized red cells

NK cells

Monoclonal and polyclonal antibodies were used to sensitize R2R2 red cells (approximately 30,000 D sites/cell) or -D- red cells (approximately 150,000 D sites/cell). Although a range of monoclonal antibodies was selected for study, an $ADCC^+$ IgG3 anti-D was unavailable, so an $ADCC^+$ human monoclonal IgG3 anti-c was used. In order to promote rosette formation with NK cells, minimum levels of sensitization with $ADCC^+$ antibodies were approximately 2000 IgG1 or IgG3 molecules/red cell (Fig. 1). $ADCC^+$ monoclonal and polyclonal were functionally indistinguishable. In contrast, the minimum levels of sensitization for rosette formation by $ADCC^-$ antibodies were 15,000 IgG1 or 4000 IgG3 molecules/red cell. Approximately 25% NK cells formed rosettes with red cells sensitized with 30,000 IgG molecules/red cell despite the finding that over 90% of NK cells bound anti-CD16 and approximately 70% formed rosettes with red cells sensitized with 30,000 rabbit IgG molecules/cell. Somewhat fewer rosettes were apparent with E-IgG1 than with E-IgG3; at 10,000 $ADCC^+$ IgG molecules/red cell, approximately 10% NK cells formed rosettes with E-IgG1 compared to 15% with E-IgG3. Red cells sensitized with polyclonal IgG2 anti-D (30,000 molecules/red cell) rosetted with 6% NK cells (single experiment). Therapeutic polyclonal anti-D, containing a mixture of IgG1 and IgG3 anti-D, promoted 11% rosette formation (10,000 IgG molecules/red cell, $n=4$).

B cells

B cells formed rosettes with human red cells only at relatively high levels of sensitization; approximately 60,000 IgG1 or 20,000 IgG3 molecules/cell (Fig. 2). $ADCC^+$ and $ADCC^-$ antibodies promoted rosette formation to the same extent. Using red cells sensitized with 140,000 IgG3 anti-D molecules/

Table 2. K-cell-mediated red lysis promoted by monoclonal and polyclonal antibodies. Specific lysis was determined as described in Materials and Methods, except for IgG2 anti-D which was tested as an affinity-purified eluate using -D- cells

Antibody	IgG concentration of neat supernatant (μg/ml)	% specific lysis				
		Neat	1/10	1/100	1/1000	1/10,000
R1D7	4·5	97	99	95	75	12
1A11	0·9	86	88	0	0	0
AB5	17·0	26	14	1	0	0
JAC10	5·0	10	0	0	0	0
1A3-3	4·5	14	15	0	0	0
FEF7	5·4	30	43	5	0	0
BB7.D4	55·0	87	54	4	1	0
IgG2	1·6	ND	9	0	0	0
Mys1	NA	ND	ND	ND	ND	ND
Mir	NA	85	94	95	23	0
Krzep	NA	ND	90	34	0	0

ND, not determined; NA, not applicable.

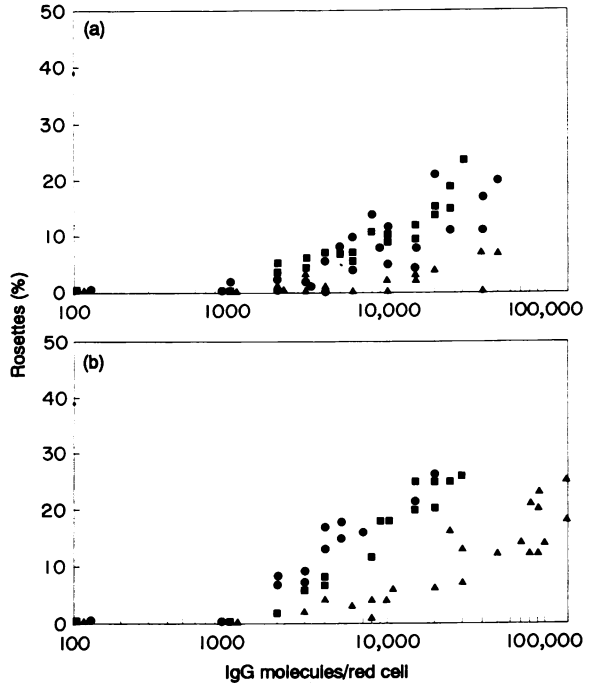

Figure 1. Rosette formation between human NK cells and red cells sensitized with IgG1 anti-D (a) or IgG3 anti-D (b). Red cells were sensitized with the following antibodies: polyclonal IgG1 or IgG3 anti-D (●), ADCC⁻ monoclonal IgG1 (AB5 and JAC10) or IgG3 (1A3-3 and FEF7) anti-D (▲), or ADCC⁺ IgG1 anti-D (1A11 and R1D7) or IgG3 anti-c (BB7.D4) (■).

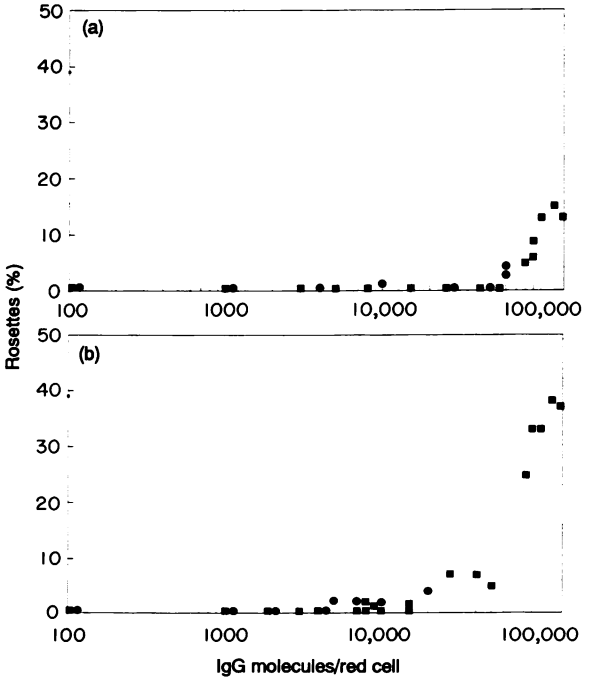

Figure 2. Rosette formation between human B cells and red cells sensitized with IgG1 anti-D (a) or IgG3 anti-D (b). Red cells were sensitized with the following antibodies: polyclonal IgG1 or IgG3 anti-D (●), or monoclonal IgG1 (AB5, JAC10, 1A11, and R1D7) or IgG3 anti-D (1A3-3 and FEF7) or IgG3 anti-c (BB7.D4) (■).

cell, approximately 35% of B cells formed rosettes. Extrapolating the data however, increased rosette formation might have been expected had higher levels of sensitization between achievable. Fewer rosettes were observed with E-IgG1 than with E-IgG3; approximately 15% of B cells formed rosettes with maximally sensitized E-IgG1. The low levels of rosette formation may have been due in part to the use of human red cells. Human, chicken and ox red cells sensitized with 30,000 rabbit IgG molecules/cell rosetted with approximately 35, 45 and 50% of B cells respectively. The zeta potential of human red cells was higher than chicken or ox cells (−1·06, −0·86 and −0·83 microns/second/V/cm respectively). Thus, the relatively high negative charge on human red cells may have contributed, at least in part, to the low level of rosette formation with B cells.

T cells

Red cells sensitized with approximately 100,000 IgG3 anti-D molecules/red cell formed rosettes with between 5 and 15% of T8 cells from different donors ($n=12$). Cytometric analyses revealed binding of anti-CD16 to 13·3±4·5 (mean±1 SD, $n=18$) of CD8⁺ cells. Although approximately 5% of T8 cells formed rosettes with E-IgG1 at 100,000 IgG molecules/cell, up to 5% T8 cells also formed rosettes with unsensitized red cells. Rosette formation with T4 cells was not observed, consistent with the binding of anti-CD16 to 1·0±0·7 (mean±1 SD, $n=18$) of CD4⁺ cells.

DISCUSSION

Earlier studies of Zupanska *et al.*[8] showed that approximately 10% of human lymphocytes formed rosettes with red cells sensitized with 20,000 IgG molecules/cell. The current study shows that approximately one-half of such rosette formation would presumably have been mediated by FcγRIII-bearing NK cells which comprised 24% (mean of four experiments) of cells before immunomagnetic separation. The ability of NK cells to rosette with red cells sensitized with fewer IgG molecules than required by FcγRII-bearing B cells reflects the 'medium and low' affinities of these receptors for monomeric human IgG.[1]

The data suggested two structural characteristics of IgG which might affect the efficiency of rosette formation with FcγR-bearing cells. First, IgG hinge region length, which presumably determines the degree to which two (negatively charged) cells must approach one another before antibody molecules can bridge between them. Thus IgG3, with the longer hinge region, was more efficient than IgG1 at promoting rosette formation with B cells. IgG3 is similarly more efficient than IgG1 at promoting monocyte rosettes[6] although in the current study it was only slightly more efficient than IgG1 in promoting rosettes with NK cells, confirming previous observations.[10,18] Second, the number of Fc–FcγRIII interactions mediated by each IgG molecule may affect the stability of rosette formation. Gergely *et al.*[18] reported that while CH3–FcγRIII interactions promoted contact between NK cells and target cells, additional CH2 domain interactions were required to activate K cells for lysis. These observations are consistent with the current study where ADCC⁺ IgG1 and IgG3 antibodies promoted NK cell rosette formation with greater efficiency than ADCC⁻ antibodies. Further, red cells sensitized with (ADCC⁻) polyclonal IgG2 anti-D promoted levels of rosette formation comparable to levels achieved with ADCC⁻ monoclonal IgG1. Thus, amino

acid differences between IgG subclasses in the region of Lys[274] might result in the failure of IgG2 to bind FcγRIII via CH2.[18,19] Further evidence for the role of both CH2 and CH3 regions in FcγRIII interactions comes from the recent observation that aglycosylated IgG antibodies with altered CH2 domain ligand-binding properties, have approximately 50% less activity in NK-cell rosette assays.[20] Together, these observations may explain the results of Zupanska *et al.*[8] who found that polyclonal anti-D sera were more efficient than some monoclonal anti-D at promoting rosettes with mixed lymphocytes; sera containing IgG1 and/or IgG3 anti-D are rarely inactive in K-cell–ADCC assays.[21]

An unexpected observation was the inability of the majority of NK cells to form rosettes with red cells sensitized with over 100,000 IgG3 anti-D molecules/red cell despite the finding that over 90% bound anti-CD16 and 70% formed rosettes with red cells sensitized with rabbit IgG. Previous studies had shown that almost all FcγRIII-bearing granulocytes may form rosettes with E-IgG3 at 35,000 IgG molecules/cell.[9] The current data were not related to the use of cells pooled from six donors for the majority of experiments since NK cells from individual donors formed rosettes with E-IgG3 (30,000 molecules/red cell) to approximately the same extent ($22{\cdot}5 \pm 4{\cdot}0\%$ rosettes, mean $\pm$ 1 SD, $n=6$). Loss of FcγRIII function resulting from the cell-isolation procedure was unlikely since levels of rosette formation using unseparated mixed lymphocytes were also consistent with only a minority of NK cells forming rosettes.[10] A similar observation was made by Zupanska *et al.*[8] who found that red cells sensitized with a 'Ripley-like' anti-C+D serum (70,000 IgG molecules/cell) did not form more rosettes with mixed lymphocytes than red cells sensitized with sera containing anti-D. Warren and Skipsey[22] reported that two distinct populations of NK cells can be distinguished in peripheral blood based on the intensity of staining with anti-CD16 antibodies, $CD16^{bright}$ and $CD16^{dim}$. Although the former population predominated, it is possible that NK cells expressing high levels of FcγRIII preferentially form stable rosettes. Alternatively, FcγRIII on some NK cells may be blocked, perhaps by serum-derived cytophilic IgG.[23]

Rosette formation with B cells was only observed at high levels of sensitization. The low affinity of FcγRII presumably necessitates the interaction of many FcγR with cell-bound IgG in order to form stable rosettes. The binding of immune complexes to low affinity FcγR also depends upon Fc valency.[24] Although the levels of red-cell sensitization required for rosette formation with B cells are unlikely to be achieved *in vivo*, FcγRII function on B cells may depend on the specificity of antigen receptors, so that cross-linking of surface Ig and FcγRII by antigen and antigen-bound IgG delivers inhibitory signals to prevent cell activation.[25]

Approximately one-third of peripheral blood B cells have been reported to bind human IgG and to form rosettes with sensitized ox red cells.[26,27] In the current study, a similar proportion of B cells formed rosettes with human red cells sensitized with either human or rabbit IgG. However, these data may underestimate the percentage of B cells expressing FcγRII since up to 50% cells formed rosettes with ox and chicken red cells (with lower zeta potentials than human red cells) sensitized with rabbit IgG, and approximately 90% B cells bound anti-CDw32 antibodies.

Anti-D sensitized red cells bound to approximately 10% of T8 cells but not to T4 cells. Although these results are in contrast with those of Vaickus *et al.*[26] who found that 28% of $CD8^+$ cells and 36% of $CD4^+$ bound human IgG, they are consistent in the current study with the binding of anti-CD16 by approximately 15% of $CD8^+$ cells and 1% of $CD4^+$ cells.

In summary, results from the current study are consistent with the role of IgG hinge length and of both CH2 and CH3 domain interactions in the formation of stable rosettes with B cells and NK cells respectively. K-cell-mediated ADCC and NK rosette assays revealed differences among human monoclonal antibodies with respect to their ability to mediate CH2/FcγRIII interactions. With the prospect of using such antibodies therapeutically, it will be important to establish the significance of this functional heterogeneity.

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