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Distinctive role of IgG1 and IgG3 isotypes in FcγR-mediated functions

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SUMMARY

Polyclonal and monoclonal anti-Rh (D) antibodies of IgG1 and IgG3 subclass were evaluated for their capacity to sensitize erythrocytes and (i) to trigger monocyte and K-cell mediated antibody-dependent cellular cytotoxicity (ADCC); (ii) to mediate binding to monocyte and lymphocyte FcγR; (iii) to stimulate phagocytosis by monocytes. All antibodies were equally effective in mediating monocyte or activated U937 cell ADCC but IgG1 was more active than IgG3 in K-cell mediated ADCC. IgG3-sensitized erythrocytes inhibited IgG1-induced lysis, suggesting that each subclass engages the same FcγR receptor but that lysis requires a further 'signal' that the IgG3 molecule can not deliver. Two monoclonal IgG3 anti-D antibodies were shown to have higher binding (two times) and phagocytic (three times) indices than IgG1 antibody for monocytes; similar differences were observed for polyclonal IgG1 and IgG3 antibodies. The same pattern was observed in an EA rosette assay when a total lymphocyte population was used; however, this difference was not seen with a B-cell depleted (T+ null cell) lymphocyte population.

INTRODUCTION

Receptors recognizing the Fc region of human IgG (FcγR) are expressed on the membrane of many cell types including monocytes, macrophages, granulocytes, K cells, B cells and some T cells. To date three distinct FcγR have been identified biochemically and with specific monoclonal antibodies (Anderson & Looney, 1986) and it has been shown that more than one FcγR may be expressed on a given cell type. Thus, monocytes express both FcRI and FcRII, neutrophils both FcRII and FcRIII, whilst B cells and T cells express only FcRII and FcRIII, respectively. Each FcγR exhibits a distinctive IgG subclass recognition specificity. This has been most convincingly demonstrated for FcRI, a high affinity receptor that binds not only complexed but monomer IgG also. The IgG1 and IgG3 isotypes bind to FcRI with a ten-fold higher association constant than IgG4, whilst binding of IgG2 is not demonstrable (Anderson & Abraham, 1980; Woof *et al.*, 1988; Kurlander & Batker, 1982). Binding to FcRII and FcRIII can only be demonstrated for complexed immunoglobulins and current knowledge depends largely on experiments performed with physically or chemically aggregated IgG myeloma proteins. These studies suggest that FcRII binds IgG1 and IgG3 with higher avidity than IgG2 and IgG4 whilst FcRIII recognizes only IgG1 and IgG3 (Dickler, 1977; Kurlander & Batker, 1982).

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FcRI on monocytes has been suggested to interact with an epitope located at the N proximal end of the CH_2 domain of IgG (Partridge *et al.*, 1986, Woof *et al.*, 1986), whilst FcγR on K cells (FcRIII), as we have demonstrated previously, possesses two interaction sites, one reacting with the CH_2 domain of target-sensitizing IgG mediating the induction of lytic signal, and the other within the CH_3 domain, being responsible for the adherence of target cells to the effectors (Sármay *et al.*, 1984, 1985).

Since these IgG-specific receptors play a critical role in immune-elimination mechanisms, it is of considerable importance to clarify the effector cells and mechanisms that may be activated within an antibody response and its dependence on the proportions of each IgG subclass of antibody elicited by target immunogen. An important and potentially illuminating system is the triggering of effector mechanisms by rhesus D red cells sensitized with anti-D antibody. The monocyte and K-cell mediated response to Rh-positive red cells is shown to be restricted to the IgG1 and IgG3 isotypes, and in *in vitro* assays many clear differences in the activity of IgG1 and IgG3 to trigger effector mechanisms have been demonstrated. Thus, sera containing mainly IgG3 anti-D antibody potentiate for EA-rosette formation of monocytes much more efficiently than those containing a high level of IgG1 (Županska, Thomson & Merry, 1986), and similar results have been obtained with monoclonal anti-D antibodies (Wiener *et al.*, 1987; Walker *et al.*, 1988). Studying the possible subclass differences in K-cell mediated ADCC function, which tend to be associated with clinical

manifestations of symptoms of haemolitic disease of newborn (HDN) (Urbaniak & Greiss, 1980), a significantly higher ADCC activity was found with sera where the IgG3 agglutination titres were higher than the IgG1 titres.

On the basis of these findings, we focused our attention on the functional role of the two preferentially binding IgG isotypes: IgG1 and IgG3. Our results show that whilst IgG1 and IgG3 bind similarly to FcRI on monocytes, IgG3-sensitized erythrocytes form rosettes and are phagocytosed with a much higher efficiency. Similar results were obtained for lymphocytes in the EA rosette assay. On the other hand, the T plus null cell fraction containing the FcRIII-positive population binds both types of sensitized red blood cells (RBC). In inducing lymphocyte (K-cell)-mediated ADCC, anti-D antibodies of the IgG1 isotype were more effective, and when target cell lysis was effected by monocyte or activated U937 cells both isotypes were equally effective.

MATERIALS AND METHODS

Separation of cells

Peripheral blood mononuclear cells (PBMC) were separated from the venous blood of healthy volunteers according to the method of Boyum (1968). Monocytes were purified by plastic adherence. Rosette formation, phagocytosis and cytotoxic activity of monocytes were tested on cells adhered to the surface of polystyrene plates. The adherent population usually contained 85% monocytes. From the non-adherent population (lymphocytes), B cells and the T plus null cell fraction were separated on a nylon-wool column (Aman, Ehlin-Henricksson & Klein, 1984). The latter cell fraction contain, as an average, 30% FcR-positive cells (detected by EA rossette assay) and was used as effector cells in the K-cell mediated ADCC system.

U937, a human monocyte-like hystiocytic cell line, was also used as effector cells in ADCC assay. The cell line was maintained in stationary culture in RPMI-1640 medium containing 10% fetal calf serum (FCS) and antibiotics. In order to trigger ADCC activity, U937 cells (5×10^5/ml) were stimulated by 50 ng/ml of phorbol-12-myristate-13-acetate (PMA; Sigma, St Louis, MO) for 24 hr (Trinchieri *et al.*, 1984).

Polyclonal anti-D antibodies

Polyclonal anti-D sera were kindly provided by Dr Gerda de Lange (Central Laboratory of The Netherlands Red Cross, Amsterdam, The Netherlands). One of the sera (No. 3417) contained IgG1 isotype (designated DG1), while the other (No. 3656) consisted mainly of IgG3 with some IgG1 contamination. This was removed by an immunosorbent method, using a monoclonal IgG1 subclass-specific antibody coupled to a CNBr-activated Sepharose-4B column (Pharmacia/LKB). The purified IgG3 anti-D was designated DG3. G1G3, a preparation containing both IgG1 and IgG3 anti-D antibodies, was obtained from National Institute of Haematology and Blood Transfusion, Budapest, Hungary.

Monoclonal anti-D antibodies

The monoclonal antibodies were produced by stable Epstein–Barr virus (EBV)-transformed cell lines (Doyle *et al.*, 1984) provided by Dr B. Kumpel (U.K. Transplant Service, Bristol, Avon, U.K.; Fc3, JoFe 2B6/1, PhT 1A3/1, CB6, JoFe 2B6/3, PhT 1A3/3) or by heterohybridomas established by fusing EBV-transformed lines with the mouse plasmacytoma cell line X63-Ag8.653 (Thompson *et al.* 1986) and provided by Dr K. Thompson (Cambridge University, Cambridge, U.K.; FOG-3, GAD-2, REG-A). Antibodies designated Fc3, JoFe 2B6/1, PhT 1A3/1 and REG-A are of the IgG1 isotype, while CB6, FOG-3, GAD-2, JoFe 2B6/3 and PhT 1A3/3 monoclonals were identified as IgG3.

Radioactive binding assays

Antibody binding to red cells determined by an indirect procedure. Human Rh-positive erythrocytes were treated with 1% papain solution for 10 min at room temperature to increase the number of D determinants per cell (Yust, Frisch & Goldsher, 1980). The samples were washed five times with phosphate-buffered saline (PBS) containing 1% FCS and 0·2% Na-azide (PBS/FCS). 5×10^6 pretreated red blood cells (RBC), in triplicates, were incubated with different dilutions of polyclonal or monoclonal anti-D antibodies for 30 min at 37°. After three washings with PBS/FCS, the cells were treated with a pan-specific mouse monoclonal anti-human IgG antibody B-33/20 (provided by G. László, Dept. Immunology, L. Eötvös University, Göd, Hungary) labelled with ^{125}I for 2 hr at 4°, then washed three times with PBS/FCS (monoclonal antibody B-33/20 has been shown previously to react with IgG1 and IgG3 isotypes to the same titre). The samples were repeatedly washed (four times) with PBS/FCS, transferred to clean tubes and the radioactivity measured.

Inhibition of the direct binding of polyclonal IgG1 (DG1) and IgG3 (DG3) anti-D antibodies. In this assay the papain-treated RBC were incubated simultaneously with different amounts of cold G1G3 anti-D IgG containing both subclasses and ^{125}I-labelled DG1 or DG3 antibodies, respectively. The samples were washed and measured as described above. The proteins were radioiodinated by the chloramine-T method (as described by Hunter & Greenwood, 1962) and were stored with bovine serum albumin (BSA) (1 mg/ml) and NaN_3 (0·02%) at 4°.

ADCC assay

The assay was performed as described elsewhere (Benczur *et al.*, 1979). Briefly, Rh-positive erythrocytes were treated with 1% papain solution at room temperature for 10 min, washed four times with sodium cytrate and labelled with 15 MBq of sodium ^{51}chromate (Amersham, Amersham, Bucks, U.K.) for 150 min at 37°, sensitized with 100 μl of the different anti-D antibodies for 60 min, then washed thoroughly with RPMI-1640 medium. 2×10^4 target cells were mixed with the effector cells at the ratio of 1 : 10, 1 : 5, 1 : 2·5 and 1 : 1·2.

In experiments examining the saturation of ADCC function, the target cells were sensitized with increasing amounts of anti-D antibody (1–500 μl/2×10^5 erythrocytes) and after washing they were mixed with the effectors at 1 : 10 ratio.

The ADCC assays were carried out in microtiter plates with U-shaped wells (Greiner Co.), except for U937 cells and monocytes which were tested in the monolayer of cells adhered to flat-bottomed plates. Each sample was tested in triplicates. The plates were centrifuged at 100 *g* for 5 min and incubated at 37° in CO_2 atmosphere for 12 hr. Fifty per cent of the supernatants were harvested and the radioactivity was mea-

sured in a gamma counter. The results were calculated as follows:

$$\text{Specific } {}^{51}\text{Cr release} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total incorporated activity}} \times 100.$$

EA rosette assay

Human Rh-positive red blood cells were sensitized with saturating amounts of anti-D antibodies for 60 min at 37° then washed three times with PBS, and suspended at 1%. Equal volumes of these sensitized erythrocyte suspensions and mononuclear cells at a concentration of 5×10^6/ml were mixed, centrifuged for 5 min at low speed then the samples were stored overnight at 4°. The percentage of rosette-forming cells was estimated by surveying at least 200 cells in each sample. Cells with three or more adherent erythrocytes were judged as rosettes.

Antibody-opsinized phagocytosis

Erythrocytes sensitized as above were added to monocytes adhered to the glass of a phagocytosis chamber (Fabry, Erdei & Gergely, 1983). Following incubation at 37° for 90 min the glass surface was washed extensively with RPMI-1640 medium, cooled to 4° and delivered under pressure from a pasteur pipette. This treatment removes erythrocytes adhering to monocytes and allows phagocytosed erythrocytes to be identified visually. By varying the focus of the microscope, the number of monocytes having internalized erythrocytes was counted and when expressed as the percentage of the total monocyte number is referred to as the phagocytic index.

RESULTS

Binding of monoclonal and polyclonal anti-D antibodies to Rh-positive RBC

Direct and indirect binding assays were performed to compare the level of sensitization of the Rh-positive RBC with monoclonal and polyclonal anti-D antibodies.

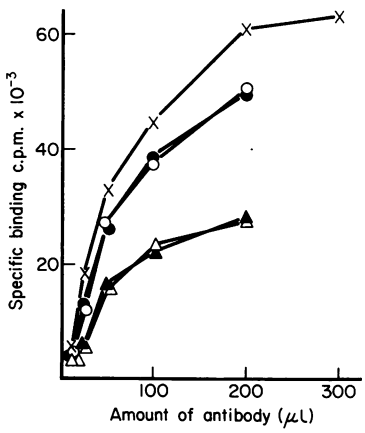

Figure 1. Binding capacity of polyclonal and monoclonal anti-D IgG antibodies to papain-treated Rh-positive RBC. Serial two-fold dilutions of monoclonal supernatants Fc3 (△), CB6 (▲) or sera DG1 (○), DG3 (●) or G1G3 (X——X) were added to red cells. Binding of anti-D antibodies was detected by a second radiolabelled anti-human IgG. (SE < 1500).

In the indirect assay bound anti-D was detected with ^{125}I-labelled monoclonal antibody (mAb) recognizing IgG1 and IgG3. The polyclonal antibodies (pAb) showed identical binding curves (Fig. 1) but saturation was not obtained for the highest input of antibody available. The monoclonal anti-D antibodies Fc3 and CB6 also showed identical binding curves near to saturation. However, it is evident that the number of antibodies bound using polyclonal reagent is approximately twice that observed with the mAb.

Binding inhibition studies with ^{125}I-labelled polyclonal anti-D antibodies DG1 and DG3 also showed that IgG1 and IgG3 reagents gave very similar levels of sensitization (data not shown). Inhibition curves obtained using iodinated DG1 and DG3 and different quantities of cold poyclonal anti-D G1G3 containing both IgG1 and IgG3 isotypes were essentially parallel.

Direct agglutination titres for each anti-D antibody were determined using papain-treated erythrocytes. The titres obtained (Table 1) were in the range of 3^6–3^{11}, allowing comparable levels of sensitization to be achieved for functional assays.

Adherence of RBC sensitized with IgG1 or IgG3 anti-D to subpopulations of mononuclear cells

Mononuclear cells from peripheral blood were further separated into plastic-adherent and non-adherent populations. Each cell population was used to study EA-rosette formation with IgG1- or IgG3-sensitized RBC. It is evident that IgG3 antibodies were more effective than IgG1 in sensitization for rosette formation and that the mAb (CB6, GAD-2) were more effective than polyclonal ones (Fig. 2).

When B cells were removed from the non-adherent cell population the T plus null cells were shown to yield essentially equivalent numbers of EA rosettes with polyclonal or monoclonal IgG1- or IgG3-sensitized red cells (data not shown).

Table 1. Direct agglutination of papain-treated human Rh-positive erythrocytes by anti-D antibodies

Antibody	Subclass	Titres ($\log_3$)
Polyclonals		
DG1	IgG1	7
DG3	IgG3	7
G1G3	IgG1+3	11
Monoclonals		
CB6	IgG3	6
Fc3	IgG1	9
FOG-3	IgG3	8
GAD-2	IgG3	6
JoFe 2B6/1	IgG1	9
JoFe 2B6/3	IgG3	6
PhT 1A3/1	IgG1	11
PhT 1A3/3	IgG3	7
REG-A	IgG1	9

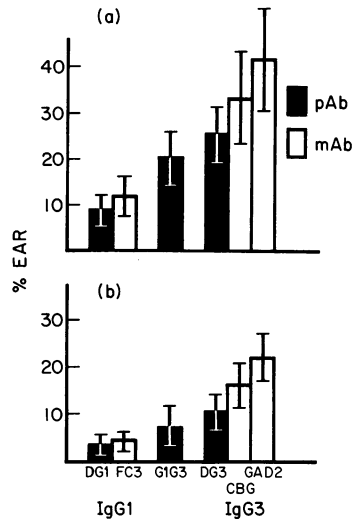

Figure 2. EA-rosette formation of (a) monocytes and (b) lymphocytes with Rh-positive erythrocytes sensitized with monoclonal (empty columns) or polyclonal (black columns) anti-D antibodies. Mean ± SE of seven experiments.

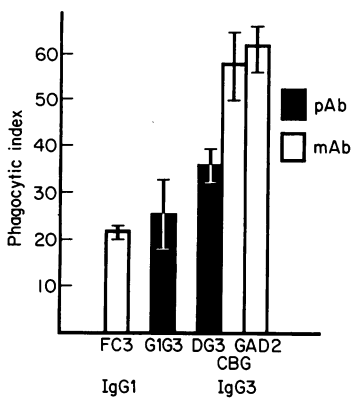

Figure 3. Phagocytosis of RBC sensitized with monoclonal (empty columns) or polyclonal (black columns) anti-D antibodies. Phagocytic indices represent the percentages of monocytes ingesting three or more anti-D-coated red cells. Mean ± SE of three experiments.

Opsonization and phagocytosis of IgG1- or IgG3-sensitized erythrocytes

Monoclonal IgG3 anti-D antibodies were shown to be more effective than monoclonal IgG1 antibody in sensitizing RBC for phagocytosis by monocytes adhered to plastic (Fig. 3); this parallels their relative efficiency in sensitization for rosette formation. The polyclonal antibodies DG3 (IgG3) and G1G3 containing both IgG1 and IgG3 isotypes were less effective than the monoclonal IgG3 antibodies.

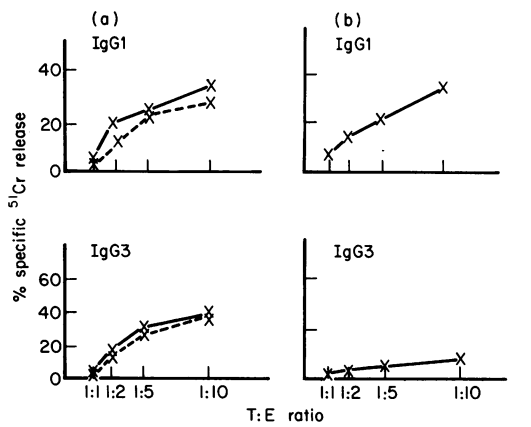

Figure 4. (a) Monocyte (——), U937 (– – –) and (b) lymphocyte-mediated cellular cytotoxicity of RBC sensitized with polyclonal IgG1 (DG1) or IgG3 (DG3) (SE < 4·5).

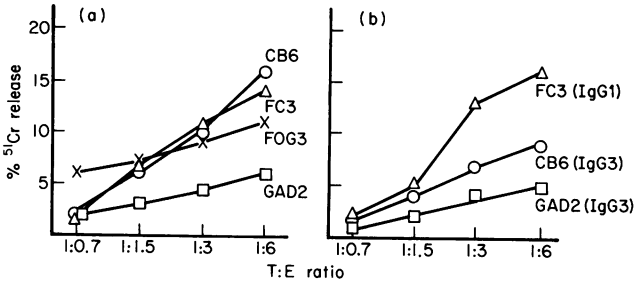

Figure 5. (a) Monocyte- and (b) lymphocyte-mediated cellular cytotoxicity of erythrocytes sensitized with monoclonal IgG1 (Fc3) or IgG3 (CB6, FOG-3 or GAD-2) antibodies (SE 2·5).

Antibody-dependent lysis of erythrocytes sensitized with IgG1 or IgG3 anti-D by plastic-adherent and non-adherent leucocytes

Target RBC were sensitized with amounts of anti-D antibody shown to give maximal binding, as demonstrated from the data of Fig. 1. Target cells sensitized to the same level with polyclonal IgG1 or IgG3 were lysed to the same extent by monocytes but when lymphocytes were used as effector cells specific lysis was observed for IgG1-sensitized targets only (Fig. 4). A similar pattern of lysis was observed for monoclonal anti-D-sensitized RBC (Fig. 5) except that the IgG1 anti-D REG-A did not sensitize for ADCC although it effectively agglutinated RBC.

In further experiments B cells (FcRII-positive population) were removed from the non-adherent leucocyte population used as effectors; also, in monocyte-mediated ADCC the monocytoid cell line U937 stimulated with PMA was used as effector. U937 cells after PMA stimulation express an elevated level of FcRII as observed by flow cytometry by the binding of mAb IV.3 (data not shown). FcRII plays a role on these cells as cytotoxic trigger molecules (Graziano & Fanger 1987).

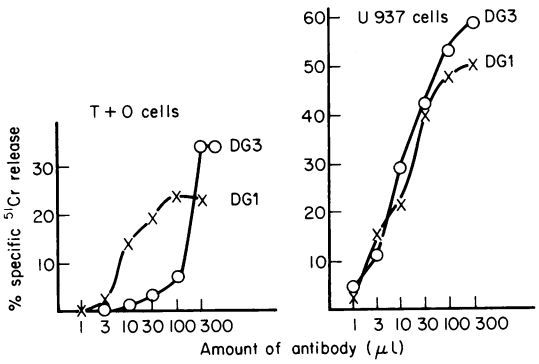

Figure 6. Antibody-dependent lysis of RBC sensitized with polyclonal IgG1 (DG1) and IgG3 (DG3) anti-Ds mediated by T plus null cells and prestimulated U937 cells at different levels of sensitization. T : E cell ratio was 1 : 20. (SE 6).

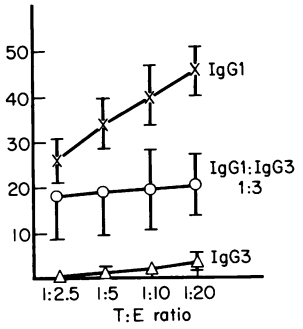

Figure 8. Comparison of lysis of erythrocytes sensitized with saturating amount of IgG1 (JoFe 3B6/1), IgG3 (JoFe 3B6/3) or with a 1 : 3 mixture of the two anti-D antibodies. T+O cells were used as effectors. Mean ± SE of three experiments.

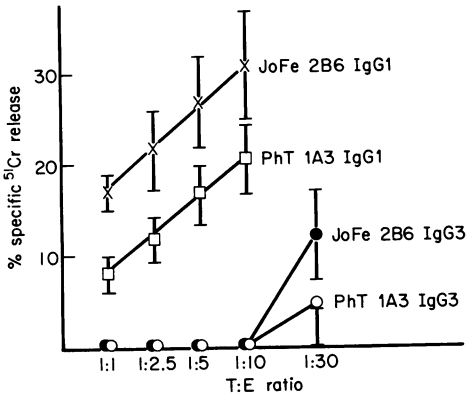

Figure 7. ADCC inducing capacity of monoclonal IgG1 (JoFe 2B6/1, PhT 1A3/1) and IgG3 (JoFe 2B6/3, PhT 1A3/3) antibodies. T+O cells were used as effectors. Mean ± SE of nine experiments.

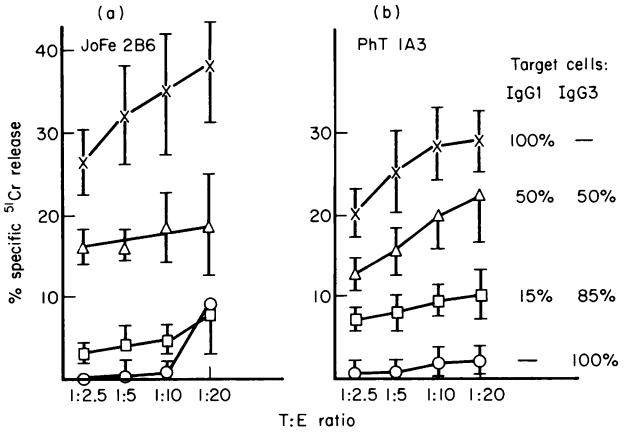

Figure 9. (a) Lysis of erythrocytes sensitized with either JoFe 2B6/1 or JoFe 2B6/3 as compared to the lysis of different mixtures of these targets. (b) A similar study with PhT 1A3/1 and PhT 1A3/3 antibodies. T+O cells were used as effectors. Mean ± SE of three experiments.

The ability of IgG1 and IgG3 anti-D to sensitize RBC for lysis by these cell populations was determined by titration of the antibody used for sensitization. The results, Fig. 6, show that at lower levels of sensitization IgG1 antibody is more efficient at inducing ADCC by the T plus null cell population than IgG3 antibody; however, at higher levels of sensitization IgG3 antibody was effective as well. When PMA-stimulated U937 cells were used as effectors, IgG1 and IgG3 antibody were equally effective at inducing lysis.

ADCC-inducing capacity of monoclonal IgG1 and IgG3 anti-D antibodies

For further experiments the following human monoclonal anti-D antibodies were selected and compared: JoFe 2B6/1, PhT 1A3/1 IgG1 antibodies and JoFe 2B6/3, PhT 1A3/3 IgG3 antibodies. The IgG1 isotype induced an efficient ADCC by T plus null cells at target : effector cell ratios from 1 : 1 to 1 : 10, while only low levels of lysis were achieved with IgG3 antibody at a ratio of 1 : 30 (Fig. 7). However, the IgG1 and IgG3 isotypes were equally effective in sensitization for rosette formation with T plus null cells.

The ability of a mixed IgG1/IgG3 population of anti-D antibody to induce ADCC mediated by T plus null cells was evaluated in two systems: first using target cells sensitized with mixed (IgG1 + IgG3) antibody, and second applying a mixed target cell population sensitized with either IgG1 or IgG3, respectively. Titration of the monoclonal antibodies showed that plateau values of lysis were achieved with 50 μl of antibodies of the IgG1 isotype whilst saturating doses of antibodies of the IgG3 isotype (200 μl supernate) failed to give comparable levels of lysis (data not shown).

The lysis of erythrocytes sensitized with saturating amounts (50 μl) of monoclonal IgG1 JoFe 2B6/1 anti-D, in the presence or in the absence of three times excess of the IgG3 (JoFe 2B6/3), demonstrates that the binding of IgG3 to target cells inhibited the IgG1-induced cytotoxicity indicating that IgG1 and IgG3 antibodies compete for epitopes in the same topographical region (Fig. 8).

A reduced level of ADCC as observed when RBC were separately sensitized with IgG1 and IgG3 antibody and a mixture of these cells at a ratio of 50–50% and 15–85% was used as targets. A progressive decrease in cytotoxicity was observed that paralleled the decrease in the number of IgG1-sensitized RBC present in the assay (Fig. 9).

DISCUSSION

In an attempt to further define the interaction between Fc receptors and IgG isotypes we have employed polyclonal and monoclonal IgG1 and IgG3 anti-D antibodies in functional assays using several subpopulations of effector cells. It was important to establish, initially, that polyclonal IgG1 and IgG3 anti-D each sensitized RBC to the same level; monoclonal IgG1 and IgG3 anti-D also gave equivalent levels of sensitization but it was approximately 50% of that achieved for polyclonal anti-D. This is presumably due to polyclonal anti-D being able to bind to multiple epitopes.

Peripheral blood leucocytes were separated into adherent (monocyte) and non-adherent subpopulations and their ability to form rosettes with anti-D-sensitized RBC was determined. Higher percentages of EA rosettes were obtained with IgG3-sensitized RBC than with the IgG1-sensitized one. Although the level of sensitization with monoclonal IgG3 had been shown to be lower than for polyclonal IgG3 or IgG1, RBC sensitized with monoclonal IgG3 gave the highest percentage of rosettes. These findings are in agreement with those of Walker *et al.* (1988) who used a panel of monoclonal IgG1 and IgG3 anti-D antibodies and showed that high percentages of EA rosettes could be formed between IgG3-sensitized RBC and U937 cells, a monocytic cell line, whilst monoclonal IgG1 anti-D antibodies were uniformly ineffective in sensitization for this activity. Weiner *et al.* (1987) have similarly shown that IgG3 antibody is more efficient in opsonizing RBC for binding to macrophages; opsonization was effected with IgG3 at a density of 100 molecules/erythrocyte whilst an equivalent activity for IgG1 was only achieved at a density of 10,000 molecules/erythrocyte. Also in the present study, IgG3 was shown to more efficiently sensitize RBC for phagocytosis. The monoclonal IgG3 anti-D antibodies were more efficient than polyclonal IgG, suggesting that the epitope specificity of the anti-D antibody may be an important contributing parameter allowing the Fc region to be more, or less, exposed and therefore available for interaction with FcγR.

A quantitative difference in the percentage of rosettes formed between IgG1- or IgG3-sensitized RBC was also observed when the non-adherent leucocyte population was used as indicator cells. This population contains T, B and K cells with the latter two subsets expressing FcγRII and FcγRIII, respectively (Anderson & Looney, 1986). However, when B cells were removed to yield a T plus null population the percentages of IgG1 and IgG3 rosettes obtained were similar. These results suggest that the FcγRII-positive B-cell population gave rosettes mainly with IgG3-sensitized RBC and thus account for the quantitative difference when the whole lymphocyte [T, B and null (K) cell] population was used.

It has been proposed that the interaction site on IgG for FcγRI is located at the *N*-proximal end of the Cγ2 domain and that the extended rigid structure of the hinge region of IgG3 results in greater accessibility of this interactive site relative to IgG1, which has a short hinge region (Partridge *et al.*, 1986; Woof *et al.*, 1986; Walker *et al.*, 1988). This structural difference results in IgG3 being more efficient in inducing the binding of sensitized RBC to monocytes. The demonstration that IgG1- and IgG3-sensitized RBC are equally effective in yielding rosette formation with FcγRIII-bearing cells is consistent with our earlier proposal that the Cγ3 domain is essential for initial binding but a subsequent Cγ2/FcγRIII interaction is responsible for the transduction of a signal determining K-cell activation (Sármay *et al.*, 1985; Sármay, Jefferis & Gergely, 1986). If the binding site for FcγRIII is in the Cγ3 domain, the hinge region will not influence accessibility and thus both the IgG1 and IgG3 isotypes allow the formation of stable rosettes.

It is of particular interest that whilst IgG1 and IgG3 each form rosettes with K cells the IgG1 isotype is more efficient in activation of the killing mechanism. However, it is consistent with our proposal that the activation signal is determined by a Cγ2 domain/FcγRIII interaction, suggesting that an explanation must be sought in tertiary structural differences resulting from the unique primary amino acid sequences of the IgG1 and IgG3 Cγ2 domains, i.e. Lys/Gln 274 and Asn/Lys 276. In contrast monocyte killing was effected by IgG1 and IgG3 antibodies, although only IgG3 sensitized for rosette formation. However, whilst stable binding may be a prerequisite for phagocytosis it may not be necessary for effective ADCC and the closer approach afforded by IgG1 antibodies may facilitate signal transfer. On the other hand, since macrophages appear to use FcγRI for ADCC and FcγRII and FcγRIII for endocytosis of immune complexes (Dougherty *et al.*, 1987), it is possible that monocyte-mediated phagocytosis and cytotoxicity may similarly be activated through different FcγR.

In previous studies it has been concluded that polyclonal IgG3 anti-D is more potent in sensitization for red cell destruction than IgG1 antibody *in vivo* and *in vitro*, but the dominant or most relevant mechanism has not been determined, i.e. phagocytosis, ADCC, O_2 radicals, etc. (Urbaniak & Greiss, 1980; Armstrong *et al.*, 1987). Others either could not demonstrate a correlation between the subclass of anti-D and its effectiveness in ADCC (Barclay *et al.*, 1985), or found IgG1 more efficient in inducing lysis (Parrinaud *et al.*, 1985). Our study demonstrates that the level of sensitization with the target isotype also affects functional activation. Thus, when RBC were optimally sensitized and high effector/target cell ratios were employed, IgG3 induced a higher degree of killing than IgG1 tested under the same circumstances. This phenomenon was only observed using polyclonal anti-D, which recognizes multiple epitopes on the D antigen. This result suggests that IgG3 above a certain density will be able to trigger FcγRIII (at least on a small population of effector cells) to mediate the lytic signal. No difference in IgG1- and IgG3-induced ADCC was observed when U937 cells were used as effectors following activation with PMA; one effect of which is to increase the density of FcγRII expression on the cell surface, as detected by monoclonal antibody, IV.3. Both FcRI and FcRII play a role in triggering ADCC mediated by monocytes (Graziano & Fanger, 1987). Unstimulated U937, however, although expressing both FcγRI and FcγRII do not lyse anti-D-coated RBC (Jones, Looney & Anderson, 1985). This suggests that the FcγRII expressed after PMA activation might be responsible for ADCC activity and that its recognition specificity for IgG1 and IgG3 is identical.

A further opportunity to definitively illustrate the difference

in the IgG1 and IgG3 isotypes for K-cell killing was provided by the monoclonal anti-D antibodies JoFe 3B6/1, JoFe 3B6/3, PhT 1A3/1 and PhT 1A3/3. When used alone only the IgG1 isotype supported killing whilst a mixture of the IgG1 and IgG3 isotypes (1 : 3) resulted in reduced killing, suggesting that there was also competition for a common epitope and that IgG3 on the RBC was able to compete with IgG1 for FcγRIII binding and activation. For K-cell killing the density of antibody on the target cell surface must be above a certain minimum to permit cross-linking of FcγRIII receptors. When IgG1 and IgG3 anti-D antibodies were used at doses in excess of that required for saturation, ADCC was reduced, suggesting that IgG3 antibody occupied FcγRIII without triggering lysis and thus preventing FcγRIII cross-linking through IgG1.

A similar result was obtained if separate populations of IgG1- and IgG3-sensitized cells were combined. Although both cells were able to bind to FcRγIII, decreased lysis was observed that paralleled the reduced number of IgG1-sensitized targets presented. This result demonstrates that IgG3-sensitized cells are not lysed in the presence of IgG1-coated ones and that the lytic mechanism operates over short distances and is concentrated to the area of functional activation (i.e. by IgG1-sensitized RBC) and thus no bystander killing is observed, even if both may bind to the same effector cells.

The difference between IgG1 and IgG3 antibodies in mediating ADCC is not restricted to anti-D antibodies, since Brüggemann *et al.* (1987) have found that the lysis of the HPB-ALL T-cell line derivatized by ^{51}Cr-NIP is more efficient with IgG1 anti-NIP chimeric antibody compared with the IgG3 isotype.

Taken together our results provide further evidence for the functional heterogeneity of FcR and for the importance of the CH_2 domain/FcγRIII interaction in the induction of K-cell ADCC.

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