Aglycosylation of human IgG1 and IgG3 monoclonal antibodies can eliminate recognition by human cells expressing FcγRI and/or FcγRII receptors

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INTRODUCTION

Interaction of IgG with cell-bound receptors for the Fc region of immunoglobulins (FcγR) is an initiating event for a wide range of biological effector functions including immune regulation, antigen clearance or elimination (Silverstein et al., 1977; Cerrotini & Bruner, 1974), and hypersensitivity reactions (Rouzer et al., 1980). Several criteria, primarily reactivity with specific monoclonal antibodies, have identified three Fc receptors (FcγRI, FcγRII and FcγRIII; previously FcRγ) and resulted in their partial characterization (Anderson & Looney, 1986). Further heterogeneity within these classes is now becoming evident (Anderson et al., 1987) particularly as a result of gene cloning studies. The three classes of FcγR may be expressed on distinctive and overlapping populations of cells and their expression has been determined for several human cell lines, namely U937, HL60, Daudi, Jurkat, and K562 (Looney et al., 1986; Jones et al., 1985; Rosenfeld et al., 1987).

The 73 kDa human FcγRI receptor binds monomeric human IgG with high affinity (Kd ~ 5.5 x 10^4 M^-1) whilst the 40 kDa FcγRII and 50–70 kDa FcγRIII bind monomer IgG weakly (Kd < 10^5 M^-1). The specificity of human FcγRII for the human subclasses has been demonstrated to be IgG1 = IgG3 > IgG4 with IgG2 essentially non-binding (Woof et al., 1986; Walker et al., 1988a). FcγRII expressed by platelets has been demonstrated to bind all four human IgG subclasses (Martin et al., 1978; Karas et al., 1982), whilst FcγRII expressed on Daudi and K562 cells has been demonstrated to bind only the IgG1 and IgG3 subclasses (Walker et al., 1989). The IgG subclass specificity of human FcγRII remains unresolved.

The FcγRII interaction site on IgG has been proposed to be located within the ‘hinge-link’ region, probably involving residues 234–238, within the Cy2 domain (Woof et al., 1984, 1986; Partridge et al., 1986). Indeed, site-directed mutagenesis within this region of a mouse IgG2b monoclonal antibody permitted binding to FcγRI (Duncan et al., 1988) whereas the unmutated IgG2b antibody did not. The interaction site on IgG for human FcγRII has yet to be determined, whilst both Cy2 and Cy3 domains are implicated in the recognition of human IgG by FcγRIII (Sarmay et al., 1986).

The role of Fc N-linked carbohydrate in interaction of IgG with effector molecules remains to be conclusively demonstrated, although several studies have shown that aglycosylation of IgG prevents its interaction with several important biological effector molecules (Nose & Wigzell, 1983; Leatherbarrow et al., 1985). Differences in the patterns of IgG N-linked Fc glycosylation have...
been implicated in rheumatoid arthritis and primary osteoarthritis (Parekh et al., 1985).

In this paper we report studies of the interaction of FcγRI expressed on U937 cells and human IgG1f and IgG3b monoclonal anti-D(Rh) antibodies produced under standard culture conditions in the absence or presence of tunicamycin. Parallel studies have been performed using IgG1f and IgG3b human chimaeric monoclonal antibodies (Brüggemann et al., 1987) specific for the hapten NIP (5-iodo-4-hydroxy-3-nitrophenethyl), allowing study of the interaction of antigen-bound glycosylated or aglycosylated IgG with the FcγRII receptor expressed on Daudi or K562 cells. This system avoids problems of interpretation associated with previous studies employing murine antibodies reacting with human FcRs.

MATERIALS AND METHODS

Monoclonal antibodies

The hetero-hybridoma cell lines REG-A and FOG-3 were produced by the fusion of human EBV-lymphocytes with the mouse myeloma cell line X63-Ag8.653 (Thompson et al., 1986). These cell lines produce human monoclonal anti-D(Rh) antibody of IgG1.G1m(f) and IgG3.G3m(b) subclasses respectively, although both express human κ light chains. Cells were maintained in RPMI 1640 (Gibco) medium containing 5% foetal calf serum (FCS). Antibody was isolated from culture supernatant by affinity chromatography using an in-house murine anti-κ constant region monoclonal antibody (72/2) coupled to glutaraldehyde-activated silicate (Boehringer-Mannheim). The production of chimaeric human IgG subclass monoclonal anti-NIP antibodies has been previously described (Brüggemann et al., 1987). Purified anti-NIP antibody was produced from culture supernatant by affinity chromatography using a NIP-hexanoate-Sepharose column (Brüggemann et al., 1987).

Tunicamycin treatment

Aglycosylated IgG1 REG-A or IgG3b FOG-3 was produced by maintaining hybridoma cells in medium containing the glycosylation inhibitor tunicamycin (Sigma) as follows. Hybridoma cells at 1 x 10^6/ml were incubated in 500 ml of medium containing 1.25 μg of tunicamycin/ml on a rotary shaker at 37 °C for 3 h. Cells were spun down and resuspended in 500 ml of fresh medium containing 1.25 μg of tunicamycin/ml and incubated for 24 h at 37 °C after which time the supernatant was harvested. Following (NH₄)₂SO₄ precipitation of the supernatant, the precipitate was resolubilized in saline and the IgG1 or IgG3 anti-D(Rh) purified by affinity chromatography as described above.

Aglycosylated human IgG1f or IgG3b anti-NIP was similarly generated using tunicamycin at 10 μg/ml.

Cell lines

The human monocytic cell line U937, human erythroblastic line K562 and Burkitt lymphoma line Daudi were maintained in RPMI 1640 medium (Gibco) supplemented with 10% FCS at 37 °C. Cells were washed three times in sterile phosphate-buffered saline (PBS) prior to use in the assays. In all cases the percentage viability of the cells assessed by their ability to exclude the dye Trypan Blue was consistently > 95%.

Inhibition of monomeric IgG–FcγRI interaction assay

Quantitative microassay of inhibition of human IgG–FcγRI binding was performed as previously described (Woof et al., 1984; Partridge et al., 1986). Briefly, monomeric polyclonal ^131I-labelled human IgG (^125I-IgG) was incubated to equilibrium with a U937 cell suspension (2 x 10^6 cells/tube) in a balanced salt solution containing 0.2% BSA and 0.1% NaN₃ at 37 °C for 1 h. Bound and free ^131I-IgG was determined following separation of supernatant and pellet by overlaying with water-immiscible oil (Versilube F.50; a gift from Dr. D. R. Burton, Sheffield, U.K.) followed by rapid centrifugation. In each assay duplicate tubes containing an excess of unlabelled polyclonal IgG (1 x 10^-5 M) gave values for non-specific binding. The inhibitor concentration generally varied in the range 5 x 10^-5 to 10^-8 M while the concentration of cells and ^131I-IgG remained constant. Assays were performed in duplicate and a control inhibition curve for normal polyclonal IgG was included in each case.

SDS/PAGE

SDS/PAGE was performed in 10% crosslinked gels under reducing conditions according to the method of Weber & Osborn (1969). Samples were prepared in 0.1 M-Tris/HCl, pH 7.0, containing 2% SDS, 2% mercaptoethanol and 10% sucrose and heated to 100 °C for 10 min prior to electrophoresis.

Red cell sensitizations

Human O+ (R1R2) red blood cells, kindly provided by Dr. D. McDonald (Blood Transfusion Service, Edgbaston, Birmingham, U.K.) were washed five times in sterile PBS and packed by centrifugation at 2600 rev./min for 5 min. Washed red blood cells were sensitized with human monoclonal anti-D(Rh) antibody as described previously (Walker et al., 1989). Sensitized cells were then washed three times in sterile PBS and resuspended to 1% suspension in PBS. For sensitization with anti-NIP MAb, washed cells were washed twice with iso-osmotic borate buffer, pH 8.3, and packed in the same buffer. Packed cells (1 ml) were incubated for 1 h at room temperature on a rotator with 9 ml of borate buffer, pH 8.3, containing 100 μg of NIP-hexanoate-O-succinimide (Cambridge Research Biochemicals, Harston, Cambridge, U.K.) dissolved in a minimum volume of dimethylformamide (Sigma). The NIP-derivatized cells were then washed four times with PBS and 200 μl of a 10% suspension of cells was incubated with 100 μl of purified anti-NIP MAb at 200, 100, 50, 25, 10 and 5 μg/ml in PBS for 1 h at 37 °C. Sensitized red cells were further washed three times with PBS and resuspended to give a 1% suspension.

Rosette assays

Rosetting of sensitized red blood cells to effector cells was performed according to the protocol described by Anderson et al. (1986) using a ratio of 100 red blood cells: 1 effector cell as previously described (Walker et al., 1988). Inhibition of rosette formation was performed by incubating the reaction mixture in the presence of 50 μl of purified IgG diluted in PBS to the concentrations indicated in the text.
Haemagglutination assay

Haemagglutination was performed in U-shaped microtitre trays (Titertek) as previously described (Lowe et al., 1982). Ascitic fluid containing murine MAb specific for epitopes on human IgG (Lowe et al., 1982; Nik Jaafar et al., 1983) (see Fig. 2) were doubly diluted from a 1:10 external dilution in Hepes buffer containing 2% FCS.

RESULTS

Preliminary experiments for each of the anti-Rh(D) secreting hetero-hybridoma cell lines (REG-A and FOG-3) established that a tunicamycin concentration of 1.25 µg/ml was optimal for secretion of aglycosylated IgG with acceptable cell mortality (results not shown). Similar experiments established that for the IgG1 and IgG3 anti-NIP secreting hybridoma cell lines the optimal concentration of tunicamycin was 10 µg/ml (results not shown). Fig. 1 shows SDS/PAGE analyses under reducing condition of the IgG affinity-purified from culture supernatant derived from each hybridoma cell line grown in the absence or presence of tunicamycin. The aglycosylated heavy chains are evident from their increased relative mobility through the gel compared to the glycosylated heavy chains (Cusheley et al., 1982). Staining of an immunoblot of the heavy chains of glycosylated REG-A but not aglycosylated REG-A by concanavalin A bound to horseradish peroxidase (Walker et al., 1987) was also consistent with aglycosylation of the heavy chain produced in the presence of tunicamycin. Differences in the mobilities of the light chains of anti-D(Rh) and anti-NIP clones probably reflects usage of different variable region genes and/or rearrangements. However, significant (~40%) of the tunicamycin-treated FOG-3 can be seen to be degraded yielding a fragment equivalent to IgG3Fc; this was accounted for in calculating antibody concentrations used for red blood cell sensitizations. Contaminating albumin and other medium components were also evident in some tracks, but were omitted from such calculations.

Analysis of red blood cells sensitized with maximal levels of anti-D(Rh) antibody or 20 µg of anti-NIP antibody in a haemagglutination assay employing a panel of murine monoclonal antibodies specific for epitopes on human Fcγ (Fig. 2) demonstrated no significant differences in the reactivity observed using glycosylated or aglycosylated human IgG1 or IgG3 antibodies. Equivalent levels of sensitization of the red blood cells with glycosylated or aglycosylated IgG were evident in the haemagglutination assay using IgG subclass-specific murine MAb (results not shown).

Significant differences were however evident when sensitized red blood cells were applied to rosette assays using U937, Daudi or K562 cell lines. Red blood cells sensitized by glycosylated FOG-3 IgG3k anti-D(Rh) formed significant (>60%) rosettes with the FcyRI+/FcyRII+ U937 cells whilst no rosetting was observed with aglycosylated FOG-3 or glycosylated or aglycosylated IgG1 anti-D(Rh) REG-A (Table 1). No anti-D(Rh) sensitization of red blood cells was capable of mediating rosette formation with the FcyRI+/FcyRII+ Daudi or K562 cell lines (results not shown). Rosettes formed between U937 cells and red blood cells sensitized with IgG3 anti-D(Rh) monoclonal antibodies CB6 or PhTIA3 (see Walker et al., 1989) could be inhibited by monomeric glycosylated IgG1 REG-A (Fig. 3a, open symbols) whilst >35-fold more aglycosylated REG-A was required to produce equivalent inhibition (Fig. 3a, closed symbols). Similar results were obtained using tunicamycin-treated IgG3b FOG-3 to inhibit FcyRI-mediated rosette formation with glycosylated FOG-3 sensitized red blood cells (Fig. 3b). The binding of REG-A by human FcγRI on U937 cells was further demonstrated in an inhibition assay measuring inhibition...
of 125I-labelled monomeric polyclonal human IgG binding (Woof et al., 1984, 1986). Glycosylated REG-A gave an almost identical inhibition curve to unlabelled polyclonal human IgG \( (K_{in} = 6 \times 10^{-9} \text{M}) \) binding inhibition activity (Fig. 4).

NIP-derivatized red blood cells sensitized with varying amounts of glycosylated IgG1 or IgG3b anti-NIP monoclonal antibodies gave significant rosettes with both the FcyRI/FcyRII Daudi (Figs. 5a and 6a) and K562 (Figs. 5b and 6b) cell lines. However, red blood cells sensitized with aglycosylated IgG1 and IgG3b anti-NIP monoclonal antibodies produced in the presence of tunicamycin gave no rosetting at any level of sensitization of the cells with either the K562 or Daudi cell lines (Figs. 5 and 6). Similar results were obtained for IgG3b anti-NIP monoclonal-antibody-sensitized red blood cells using the FcyRI/FcyRII U937 cells (Fig. 5c).

Significant inhibition of rosette formation between Daudi cells and glycosylated IgG3b anti-NIP-sensitized red blood cells could be achieved using glycosylated IgG3b at concentrations > 10^{-6} \text{M} whilst no significant inhibition was evident using the aglycosylated IgG3b at concentrations > 10^{-5} \text{M} (Fig. 7).

**Fig. 2. Diagramatic representation of the location of epitopes recognized by the panel of murine MAb specific for IgG Fc epitopes**

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**Table 1. Rosetting between U937 and red blood cells sensitized with normal or tunicamycin-treated human anti-D(Rh) monoclonal antibodies**

<table>
<thead>
<tr>
<th>Sensitization</th>
<th>Rosette formation (( % \pm \text{S.E.M., } n = 6 ))</th>
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<tbody>
<tr>
<td>REG-A (IgG1f)</td>
<td>0</td>
</tr>
<tr>
<td>REG-A* (IgG1f)</td>
<td>0</td>
</tr>
<tr>
<td>FOG-3 (IgG3b)</td>
<td>62 \pm 3</td>
</tr>
<tr>
<td>FOG-3* (IgG3b)</td>
<td>1 \pm 1</td>
</tr>
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*FOG-3 and REG-A, are antibodies produced in the presence of tunicamycin.
DISCUSSION

Several workers have investigated the ability of aglycosylated IgG, from several animal species, to interact with biologically important effector molecules and subsequent effector function activation. Two approaches have been used: removal of carbohydrate by glycosidase treatment (Williams et al., 1973; Koide et al., 1977; Winkelhake et al., 1980) or culturing IgG-secreting hybridoma cells in the presence of tunicamycin which prevents N-linked carbohydrate attachment at Asn-297 in the Cy2 domain (Nose & Wiggell, 1983; Leatherbarrow et al., 1985). Using the latter approach, aglycosylated murine monoclonal IgG2a and IgG2b anti-hapten monoclonal antibodies have been demonstrated to exhibit impaired complement activation, loss of binding to murine macrophage Fc receptors and ability to activate ADCC (Nose & Wiggell, 1983), a lowered rate of Cl activation and a >50-fold decrease in the apparent binding constant of aglycosylated IgG2a to the human FcγRI receptor on U937 cells (Leatherbarrow et al., 1985).

In this paper we describe the interaction of aglycosylated human IgG1 and IgG3b anti-D(Rh) or anti-NIP monoclonal antibodies with human FcγRI and FcγRII receptors expressed on U937, Daudi and K562 cell lines. Aglycosylated human monoclonal antibodies were produced by culturing hybridoma cells in the presence of 1.25 or 10 μg of tunicamycin/ml (see the Materials and methods section).

Analysis of red blood cells sensitized with glycosylated or aglycosylated human IgG1 or IgG3b monoclonal antibodies in a haemagglutination assay using a panel of murine MAbs specific for epitopes within the Cy2, Cy3 or Cy2/Cy3 interface region of IgG Fc (Fig. 2) demonstrated no differences in reactivity between the glycosylated or aglycosylated IgG. This suggests that tunicamycin treatment has not resulted in significant conformational changes within the Fc region. In contrast, red blood cells sensitized with aglycosylated FOG-3 IgG3b anti-D(Rh) antibody were unable to form rosettes with the FcγRI/FcγRII* U937 cells whilst cells sensitized with glycosylated FOG-3 antibody formed significant (>60%) rosettes (Table 1). We have previously demonstrated that rosetting of human IgG anti-D(Rh)-sensitized red blood cells is an essentially FcγRI-mediated event and, without γ-interferon stimulation or bromelain treatment, U937 cells only form rosettes with IgG3-sensitized red blood cells; this is
postulated to be due to its extended hinge region (Walker et al., 1988). Monomeric IgG1 or IgG3 can however be demonstrated to bind to human FcγRI due to its high affinity for monomer either by inhibition of IgG3 anti-D(Rh) mediated rosetting or by competition of 125I-labelled monomeric IgG binding (Woof et al., 1984, 1986). Inhibition of rosette formation by both the aglycosylated IgG1 (REG-A) and IgG3b (FOG-3) anti-D(Rh) antibodies exhibited a >35-fold lower inhibitory capacity than by the glycosylated forms (Fig. 3). Inhibition of binding of monomeric 125I-labelled polyclonal human IgG to U937 FcγRI was reduced by >50-fold for the aglycosylated form of IgG1 REG-A ($K_{i}$ of 33 M) (Fig. 4) compared with unlabelled polyclonal IgG or glycosylated REG-A ($K_{i}$ of 6 x 10^{-8} M). These values compare favourably with those obtained by Leatherbarrow et al. (1985) for aglycosylated murine IgG2a monoclonal antibody. Residual binding of the aglycosylated form of murine IgG2a was suggested to result from contamination of the preparation (by <3%) with native glycosylated murine IgG2a. Within our study, significant contamination of the aglycosylated REG-A by glycosylated REG-A is evident (Fig 1, lanes 1 and 2), but no similar contamination is observed for the aglycosylated forms of FOG-3 or IgG1 or IgG3b anti-NIP monoclonal antibodies (lanes 3–8). It is therefore possible that aglycosylation of human IgG1 or IgG3 results in a lowered binding affinity (rather than abolition) of these forms to human FcγRI similar to the observation of a reduction in the affinity of C1q binding to aglycosylated murine IgG2a (Leatherbarrow et al., 1985). The possibility that Fc carbohydrate residues directly interact with the human FcγRI receptor has been raised by several workers; however, as yet there remains no definitive evidence (for review see Burton, 1985). Recently, the involvement of residues Leu-234–Pro-238 in the human FcγRI interaction site on IgG (Woof et al., 1986) has been confirmed by site-directed mutagenesis. A monoclonal murine IgG2b anti-NIP

Fig. 6. Rosette formation between red blood cells sensitized with glycosylated IgG1 anti-NIP (open symbols) or tunicamycin-treated IgG1 anti-NIP (closed symbols) with (a) Daudi cells and (b) K562 cells

Fig. 7. Inhibition of rosette formation between red blood cells sensitized with native IgG3b anti-NIP antibody and Daudi cells by native glycosylated IgG3b anti-NIP (open symbols) and tunicamycin-treated IgG3b anti-NIP (closed symbols)
protein, normally not recognized by human FcγRI, has been engineered to give a Glu/Leu interchange at position 235 (giving the same sequence as murine IgG2a bound with high affinity by human FcγRI), producing a product recognized with high affinity by human FcγRI (Duncan et al., 1988).

It is of interest that whilst the site recognized by FcγRI is substantially altered in aglycosylated IgG we could not detect loss of epitope expression using a large panel of murine monoclonal antibodies, including antibodies previously shown to inhibit Fcγ-FcγRII interactions (Woof et al., 1986; Partridge et al., 1986). This suggests that aglycosylation results in subtle conformational changes within the Cy2 domain which can have an important effect on local sites. The direct involvement of the carbohydrate moiety in FcγRI recognition is unlikely because the sugar residues are essentially ‘buried’ in the quaternary structure (Burton, 1985); this is evidenced by the resistance to removal by endoglycosidases.

The site of interaction of human FcγRII on IgG has as yet not been delineated; however, the data presented herein suggest that similar conformational restraints are imposed upon FcγRII interaction with IgG. Both glycosylated human IgG1 and IgG3b anti-NIP-sensitized red blood cells form rosettes with the FcγRII/-FcγRII- Daudi and K562 cells, the degree of rosette formation being related to the IgG concentration on the cell surface (Walker et al., 1989; the present paper). Examination of the rosetting of red blood cells sensitized with equivalent amounts of aglycosylated IgG1 and IgG3 reveals that removal of Fc carbohydrate abolished FcγRII-mediated rosette formation (Fig. 5). Similar results were obtained if glycosylated and aglycosylated IgG3b were applied to rosette inhibition (Fig. 7), i.e. inhibition was observed with glycosylated but not aglycosylated IgG3b. Since aglycosylation of murine IgG2b has been demonstrated not to affect binding of staphylococcal protein A, which requires integrity of the Fc Cy2/Cy3 interface region (Nose & Wigzell, 1983), it would suggest that FcγRII recognizes a site within the Cy2 domain.

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