HUMAN MONOCLONAL IgG ISOTYPES DIFFER IN COMPLEMENT ACTIVATING FUNCTION AT THE LEVEL OF C4 AS WELL AS C1q

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Monoclonal antibodies have attracted much interest as agents to kill unwanted cells for therapeutic purposes. A limitation to the scope of mAb therapy is the "foreignness" of mouse and rat antibodies which elicit an antiglobulin response with its attendant problems. Recombinant DNA technology has made it possible to "humanize" therapeutic mAbs (1-3), which in part should reduce this problem. However, the maximum benefit of humanized antibodies will depend on their ability to activate effector mechanisms, such as complement- or cell-mediated killing.

To date, most of the available knowledge about the functions of the human isotypes has come from studies of myeloma proteins (4-6), which in general do not bind to identified antigens. Therefore, although the binding of C1q in solution has been extensively studied (7, 8), there had, until recently, been no definitive experiments to compare the lytic ability of the human isotypes. However, such studies were made possible by the construction of a matched set of chimeric antibodies (9). These consist of the same mouse light chain and a heavy chain assembled from different human constant regions each joined to the same mouse variable region, specific for the hapten 5-iodo-4-hydroxy-3-nitrophenacetyl (NIP).[1] The use of identical V regions means that the effect of isotype can be studied independently of any effects due to differences in antigen specificity or affinity. The NIP hapten can be attached to red cells and thus the whole pathway of complement activation can be studied from C1 binding and activation through C4, C2, and C3 binding to assembly of the membrane attack complex and cell lysis.

Among the four IgG antibodies, the IgG3 was the most efficient for C1q binding, consistent with previous findings for myeloma proteins. Suprisingly, however, the IgG1 antibody was much more efficient for cell lysis with human complement (9). To exploit the full potential of complement lysis in therapy, it will be important to understand the reason for this paradox, and to use this knowledge to engineer better antibodies.

Here we examined human IgG1, IgG2, IgG3 (including two different allotypes), IgG4, and IgE for their abilities to activate key stages of the complement cascade,

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[1] *Abbreviations used in this paper:* NIP, 5-iodo-4-hydroxy-3-nitrophenacetyl; NIP-RBC, human red blood cells coupled with NIP-kephalin.

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C1q binding, C1 binding and activation, C4 activation, and deposition of C4 and C3 on the cell membrane. A new chimeric antibody consisting of the rat IgG2b constant region joined to the same mouse anti-NIP V region was also included. This is the optimum rat IgG for fixing human complement and cell lysis (10, 11) and we have found that at least one rat IgG2b (CAMPATH-1G) (12) is very effective for cell depletion in vivo. Thus, rat IgG2b provides a standard against which the human isotypes can be compared. We show that the lytic ability of the human IgG1 is greater than rat IgG2b and that the critical advantage of human IgG1 over human IgG3 occurs at the stage of C4 activation.

Materials and Methods

Cell Handling Medium. Unless otherwise stated, cells were suspended and washed in Iscove's modified Dulbecco's medium, buffered with *N*-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid (Hepes), and containing 1% BSA. This will be referred to simply as medium.

Construction and Purification of Chimeric Antibodies. The detailed construction of the chimeric human antibodies has been previously described (9). A plasmid (pSV-V_{NP}) containing the V_H gene of a mouse anti-NIP antibody (3) was used to insert various human C_H genes downstream of the V_H. The plasmids were transfected into the mouse plasmacytoma J558L (13), which secretes a λ1 light chain, but expresses no heavy chain of its own. The endogeneous light chain complements the transfected heavy chain to yield a NIP-specific antibody. A chimeric rat IgG2b antibody was constructed using the same plasmid and a previously isolated rat IgG2b gene (14). The chimeric antibodies were purified from culture supernatant by affinity chromatography on a column of NIP-caproate Sepharose as described before (9).

Antibodies to Human Complement Components. A polyclonal sheep antiserum to human C4 was purchased from Serotec Ltd., Oxford, UK (catalog no. AHP 032X/Y). In specificity checks by the manufacturer it reacted with only the C4 component. A rat IgG2a mAb (clone 3) to the C3d fragment of human C3 (15) was the gift of Dr. R. Harrison and Professor P. J. Lachmann (Mechanisms in Tumour Immunity Unit, MRC Centre, Cambridge, UK). It was purified from ascitic fluid by precipitation with ammonium sulphate to 50% saturation followed by ion-exchange chromatography.

Radiolabeling of Antibodies. Antibodies to C4 and C3d were radiolabeled by the iodogen method (16). Between 0.5 and 1 mCi of ^{125}I was used to label ~1 mg of total protein. Labeled antibody was purified by gel filtration on Sephadex G75 and stored at 4°C in PBS containing 0.05% sodium azide. Control experiments were carried out to determine the percentage of the labeled material that could bind to cells. A small amount of antibody (0.4 μg/ml) was incubated with a large excess of cells on which complement components had been activated. Under these conditions, 4% of the polyclonal anti-C4 antibody was bound to the cells (consistent with the manufacturer's estimate of 5% specific antibody) and 65% of the anti-C3d mAb. Despite the relatively low activity of the polyclonal anti-C4 antiserum, positive binding could readily be distinguished from background binding (0.1%) to cells incubated without the primary sensitizing antibody (see also the legend to Table I). The ^{125}I-anti-C3 and ^{125}I-anti-C4 were titrated to determine what concentrations were needed to saturate the cell-bound C3 or C4.

Complement Components and Sera. ^{125}I-labeled C1q and C1 were the generous gifts of Dr. N. Hughes-Jones and B. Gorick (M.I.T.I. Unit, MRC Centre, Cambridge, UK). They were isolated from human serum (17, 18) and labeled with ^{125}I by the lactoperoxidase method (19). Purified C1 inhibitor and C4-deficient guinea pig serum were the gifts of Dr. R. Harrison. Serum from an individual genetically deficient in C6 was the gift of Dr. A. Orren (University of Cape Town, South Africa). Its hemolytic activity with respect to all other complement components was confirmed by addition of purified C6 (a gift of Dr. A. Galvani, M.I.T.I. Unit). This gave a similar hemolytic titer to normal serum. Normal human serum was obtained from defibrinated blood of healthy volunteers. It was either used fresh or stored at −70°C. Guinea pig serum was obtained from clotted blood. Cytotoxic antibody was removed by two

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cycles of absorption on human RBC, on ice, in the presence of EDTA, followed by restoration of Ca^{2+} to 10 mM, and the serum was stored at −70°C.

Preparation of NIP-coupled RBC. Human group O red cells (4–6 wk since donation) were obtained from the Blood Transfusion Service, Cambridge, UK. Red cells were washed extensively with PBS and coupled with NIP-kephalin (a gift of Dr. U. Weltzien, University of Freiburg, Federal Republic of Germany) as previously described (20).

Complement-mediated Lysis. NIP-RBC were labeled with sodium [^{51}Cr]chromate and washed twice. They were resuspended in medium at 10^7 cells/ml and 50 µl of the suspension was added to round-bottomed microtiter plates containing 50 µl of an appropriate dilution of antibody (0–200 µg/ml). After incubation for 10 min at room temperature, 100 µl of a dilution of complement was added. Human serum was used at a final concentration of 25%, and guinea pig serum at a final concentration of 5%. The suspensions were incubated at 37°C for 30 min, then centrifuged at 100 *g* for 2 min, and 100 µl of the supernatants were collected for measurement of released radioactivity. Controls with no antibody were used to measure the spontaneous radioactivity release. The percentage specific ^{51}Cr release was calculated using the formula: Percent specific release = 100 × [(test − spontaneous)/(total − spontaneous)].

C1q Binding by Antibody-coated Cells. Antibodies and C1q were centrifuged at 15,000 *g* for 10 min before use in order to remove any aggregates. Washed NIP-RBC (20 µl at 2–4 × 10^8 cells/ml) in PBS containing 1% BSA were incubated with saturating amounts of antibody (10 µl at 200 µg/ml) then ^{125}I-C1q (10 µl at 10–60 µg/ml) was added. The suspension was rotated at 37°C for 1 h, then bound and free ^{125}I-C1q were separated by centrifuging each reaction mixture through 150 µl of oil with density 1.028 g/ml (a mixture of four parts di-*n*-butyl phthalate and one part dinonyl phthalate) in microfuge tubes. The cell pellets were separated by clipping off the bottom of the tubes and the radioactivity in the "bound" and "free" fractions was determined. Specific binding of C1q was determined by subtraction of the amount nonspecifically bound in the absence of antibody (<1%).

C1 Activation by Antibody-coated Cells. This was also measured using ^{125}I-labeled material, but electrophoretic analysis was necessary to determine the amounts of the three subcomponents bound and their state of activation. Antibody (50 µl at 250 µg/ml) was added to a suspension of NIP-RBC in medium (150 µl at 2–4 × 10^8/ml) and allowed to bind at 37°C. After 10 min, 50 µl of ^{125}I-C1 (35–80 µg/ml) was added, mixed thoroughly, and immediately the first sample (50 µl) was removed and placed on ice to stop the activation reaction. In some experiments, purified C1 inhibitor (10 µl at 400 µg/ml) was added to the reaction mix before addition of C1 (80 µg/ml). The incubation was continued at 37°C and other samples were removed at various times. All subsequent procedures were carried out on ice to prevent further activation. Each sample was centrifuged, and the supernatant was removed. The cells were then lysed by the addition of 100 µl of one-tenth strength medium (diluted in water), and the membranes were pelleted by centrifugation at 15,000 *g* for 10 min. The supernatant was removed, and the membranes were resuspended and analysed by SDS-PAGE on a 12% gel under reducing conditions, followed by autoradiography. Activation of C1r and C1s involves proteolytic cleavage of the 83-kD precursors to 56-kD and 27-kD fragments (Fig. 3). These individual bands were cut out of gels and their radioactivity was measured to determine the amount of C1r and C1s in the native (83-kD) form, and the activated (56+27-kD) form (18).

C4b and C3b Binding. C4b and C3b binding were measured using ^{125}I-anti-C4 and ^{125}I-anti-C3 antibodies. NIP-RBC (20 µl and 5 × 10^7–10^8/ml in medium) were mixed with 10 µl of antibody (200 µg/ml), and 10 µl of C6-deficient serum was added. C6-deficient serum was used so that the cells would not be lysed. The suspension was incubated at 37°C for 30 min then the cells were washed twice and 40 µl of a saturating concentration of ^{125}I-anti-C3 or ^{125}I-anti-C4 was added. The mixture was incubated for 30 min at room temperature and then bound and free radiolabel were separated by centrifuging each reaction mixture through oil as above. In some experiments the cells were washed a further three times and incubated in medium with or without 0.1 mM NIP-caproic acid for 10 min on ice before centrifugation through oil. This made it possible to measure whether C4b or C3b were bound to the cells or the antibody, since control experiments showed that the anti-NIP antibodies could be eluted by this treatment (Table II).

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C4 Activation. Activation of C4 in the fluid phase by cell-bound C1s was measured by its ability to complete a hemolytic assay consisting of sensitized erthyrocytes plus C4-deficient serum. The assay was carried out in two stages. First, NIP-RBC (200 µl at 2 × 10^9/ml in medium) were mixed with 50 µl of C6-deficient serum on ice and 12-µl aliquots were mixed with 4 µl of antibody (200 µg/ml). The samples were incubated at 37°C for various times to allow complement activation to occur. Activation was stopped by addition of 300 µl ice-cold medium and the cells were pelleted by centrifugation (3,000 *g* for 5 min) at 4°C. In the second stage the supernatants were assayed for residual C4 hemolytic activity. Sheep erythrocytes sensitized with an IgM anti-Forssmann antibody were labeled with ^{51}Cr as above, then aliquots (50 µl, 10^7 cells/ml) were added to serial dilutions (50 µl) of the test supernatants in microtiter trays. The reaction was completed with 100 µl of C4-deficient guinea pig serum (diluted 1:10 with medium) and incubated for 45 min at 37°C. Released radioactivity was measured and the dilution of test supernatant that gave half-maximal lysis was calculated by nonlinear regression analysis.

Results

Complement-mediated Hemolysis. Lysis of NIP-RBC was measured using both human and guinea pig complement. With human complement (Fig. 1 *a*) the human IgG1 was much more efficient than any other isotype. Some lysis was seen with human

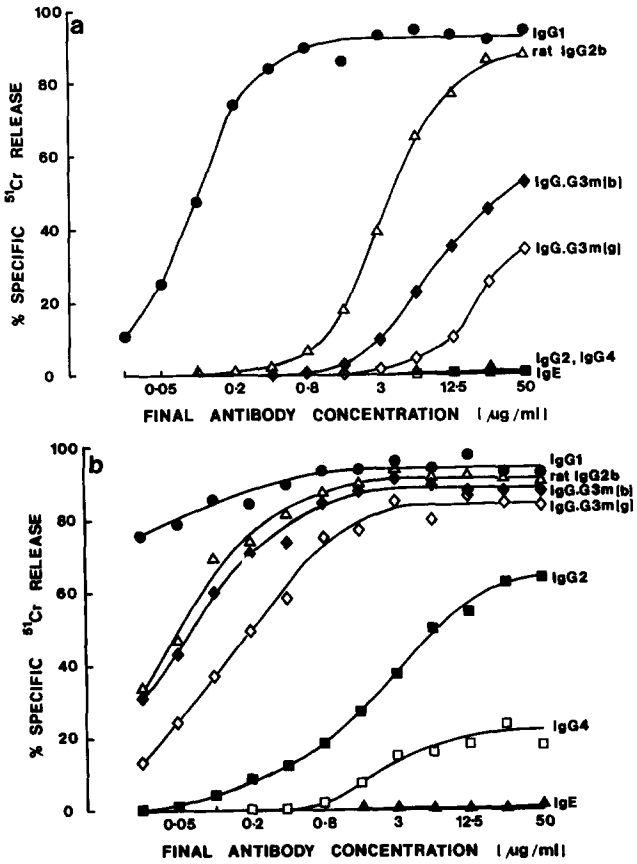

FIGURE 1. Complement-mediated lysis of NIP-RBC sensitized with different chimeric anti-NIP antibodies. Lysis was measured by a ^{51}Cr-release assay using either autologous human complement at a final concentration of 25% (*a*) or absorbed guinea pig complement at a final concentration of 5% (*b*).

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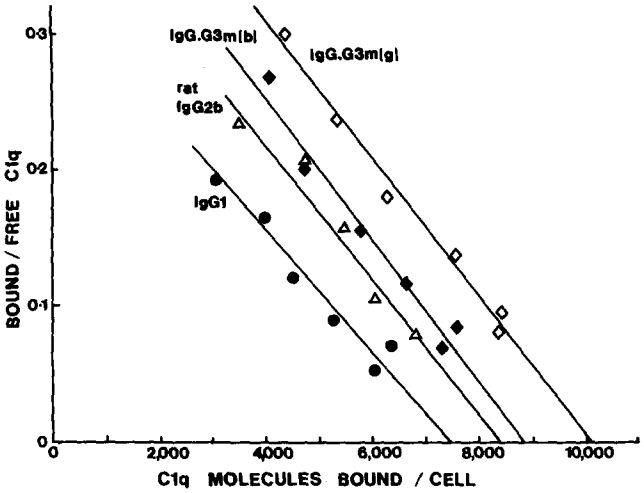

FIGURE 2. Scatchard plot of binding of C1q to NIP-RBC sensitized with different chimeric anti-NIP antibodies. NIP-RBC were sensitized with saturating amounts of anti-NIP antibodies then incubated with ^{125}I-C1q. The amount of C1q bound and free was measured after centrifuging the cells through oil.

IgG3 antibodies, but their titers were 100–500-fold less than that of the IgG1 antibody. The rat IgG2b was intermediate between the IgG1 and IgG3 antibodies. Human IgG2, IgG4, and IgE antibodies were ineffective.

With guinea pig complement the same hierarchy was obtained but all antibodies that were previously found to be lytic showed greater efficiency. Heterologous complement is generally more efficient for lysis as species-specific cell-membrane factors inhibit complement activation on the homologous cell membrane (21–26). Under the more sensitive conditions provided by the use of heterologous complement, lysis could be obtained with the IgG2 antibody, and to a lesser extent by the IgG4, although the IgE remained nonlytic.

C1q Binding. A systematic study of various stages of the complement cascade was undertaken, beginning with the first component of the classical complement pathway, C1q, in order to identify the component that determines the greater lytic efficiency

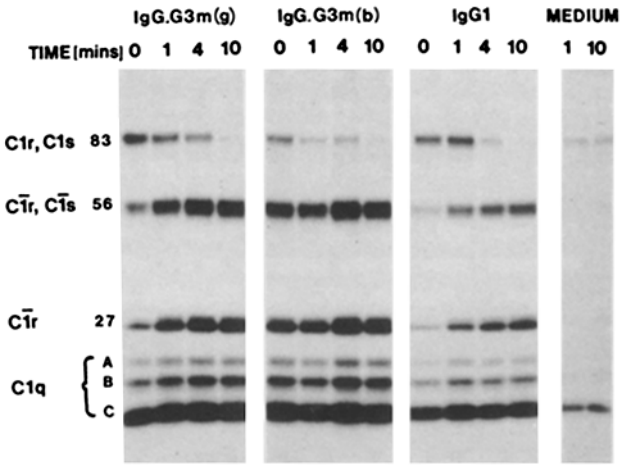

FIGURE 3. Time course of binding and activation of C1 on NIP-RBC sensitized with different chimeric antibodies. NIP-RBC were sensitized with saturating amounts of anti-NIP antibodies and incubated with ^{125}I-C1. Samples were analyzed on a 12% SDS-polyacrylamide gel to determine the fraction of the various subcomponents bound and the rate at which native (83 kD) C1r and C1s was cleaved to the 56-kD and 27-kD fragments.

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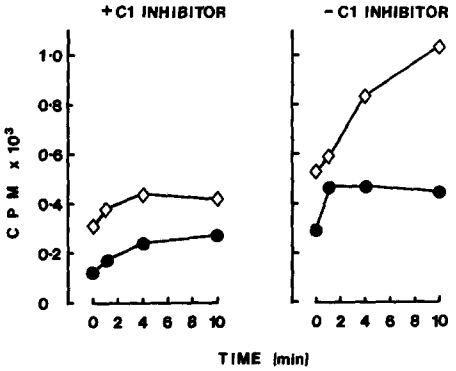

FIGURE 4. Effect of C1 inhibitor on activation of C1. NIP-RBC were sensitized with IgG1 (●) or IgG.G3m(g) (◇) antibodies and incubated with ^{125}I-C1 in the presence or absence of C1 inhibitor. Samples were analyzed on a polyacrylamide gel as in Fig. 3. The summed radioactivity in the 56-kD and 27-kD fragments is plotted as a measure of activated C1.

of the IgG1. Binding of ^{125}I-C1q was determined over a range of C1q concentrations and a Scatchard analysis (27) was performed (Fig. 2). This allows estimation of the functional affinity constants of binding (indicated by the gradient of the straight line plot), as well as the number of C1q binding sites per cell (which is the intercept on the *x* axis). The IgG3 antibodies appeared to provide more C1q binding sites per cell (~10,200 for the IgG.G3m[g] and 8,900 for the IgG.G3m[b]), than did the IgG1 antibody (~7,600). The rat IgG2b was intermediate (~8,500). However, the affinity of C1q binding (1.3–1.6 × 10^8/M) was very similar for each antibody. In other experiments using a different batch of NIP-RBC (not shown) similar results were obtained, but the IgG.G3m(g) antibody then gave ~14,500 C1q binding sites per cell and the IgG1 gave only 8,300 C1q sites. In all cases, C1q binding by the IgG2, IgG4, and IgE antibodies was undetectable. The amount of C1q bound, although contributory, was clearly an insufficient explanation for the degree of lysis seen.

C1 Binding and Activation. The IgG1 and IgG3 antibodies were compared for their ability to bind C1 and activate C1r and C1s by cleavage of the 83-kD precursors to 56-kD and 27-kD fragments (Fig. 3). Again, the IgG3 antibodies gave more C1 binding than the IgG1. Quantitative analysis (by measurement of radioactivity in bands cut out of the gels) showed that both IgG3 antibodies bound about twice as much C1 as did the IgG1 antibody. In each case nearly all of the bound C1 was activated within 10 min. Therefore, the superior lytic efficiency of the IgG1 antibody could not be explained by its ability to bind or activate C1.

It was possible that C1 activation in whole serum would be different because of the presence of C1 inhibitor, which might be able to gain better access to C1 bound to IgG3 due to the unusually long hinge of that antibody. Therefore, a similar experiment was carried out to measure C1 binding and activation in the presence of C1 inhibitor. (The molar ratio of C1 inhibitor to C1 was adjusted to be about the same as that in normal serum.) For both IgG1 and IgG3 antibodies the extent of activation was reduced (Fig. 4). However, even in the presence of C1 inhibitor the IgG3 antibody still gave more activated C1 than the IgG1 did. No additional bands were detected on the gel corresponding to a covalent complex of C1 and C1 inhibitor (~135 kD).

C4 and C3 Fixation. After activation of C1, the next step in the classical complement pathway is the activation of C4 after proteolytic cleavage by C1s. The major fragment, C4b, can attach to a nearby acceptor molecule through a covalent thiolester

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TABLE I
Amount of C4b and C3b Bound to NIP-RBC Sensitized with Different Antibodies

Chimeric anti-NIP antibody	C4b bound	C3b bound
	cpm	*cpm*
Human IgG1	2,860	8,670
Human IgG2	0	75
Human IgG.G3m(b)	1,190	4,419
Human IgG.G3m(g)	926	3,657
Human IgG4	0	0
Human IgE	0	113
Rat IgG2b	1,850	9,937

Binding of C4b and C3b was measured using ^{125}I-anti-C4 and ^{125}I-anti-C3. The bound radioactivity was measured after separation of the cells from free labeled antibody by centrifugation through oil. The results are means of duplicate samples from a representative experiment. The background radioactivity of cells incubated without sensitizing antibody has been subtracted from each sample (289 cpm for C4b and 583 cpm for C3b).

bond. The amount of C4b fixed on the cell surface was measured using ^{125}I-anti-C4 antibody. The relative amount of C4 bound by various isotypes followed a quite different hierarchy from that observed for C1 binding (Table I). The IgG1 antibody caused considerably more C4b binding than did either of the IgG3 antibodies, while the rat IgG2b antibody was intermediate between these two. No C4b binding was detected with IgG2, IgG4, or IgE antibodies. The hierarchy for C4b binding correlated well with the lysis results.

C4b fixed on the cell surface interacts with C2 to form the classical pathway C3 convertase (C4bC2b). This in turn cleaves C3 to C3b, allowing its fixation on the cell membrane through a covalent thiolester bond in a similar way to C4b. C3b binding was measured using an mAb to C3. The amount of C3b bound correlated well with the degree of lysis (Table I). The order of efficiency for both C4b and C3b binding was human IgG1 > rat IgG2b > human IgG.G3m(b) > human IgG.G3m(g). These results were also confirmed using C6-deficient serum trace labeled with ^{125}I-C3 to measure C3b binding direct (data not shown).

Thus, the major difference between human IgG1 and IgG3 antibodies involved a stage between the activation of C1, and the fixation of C4b on the cell membrane. To exclude possible artefacts that could arise if, for example, some fraction of the antibodies were inactive, we studied the binding of C1q, C1, C4b, C3b, and lysis simultaneously over a range of antibody concentrations (Fig. 5). Only one IgG3 (IgG.G3m[g]) was used as they both appeared similar for C1 binding (Fig. 2) and C4b binding (Table I). Over the whole range of antibody concentrations, the ratio of C4b/C1 molecules bound was ~10–12 times greater for the IgG1 antibody compared with the IgG3 antibody (Fig. 5). Likewise for the ratio of C3b/C1 molecules where the difference was, if anything, slightly greater.

C3b and C4b Bind to the Cell Membrane. Some studies have suggested that both C4b and C3b bind efficiently to antibody molecules (28–31). We therefore investigated whether C4b and C3b attached to the antibody or to the cell membrane. To do this,

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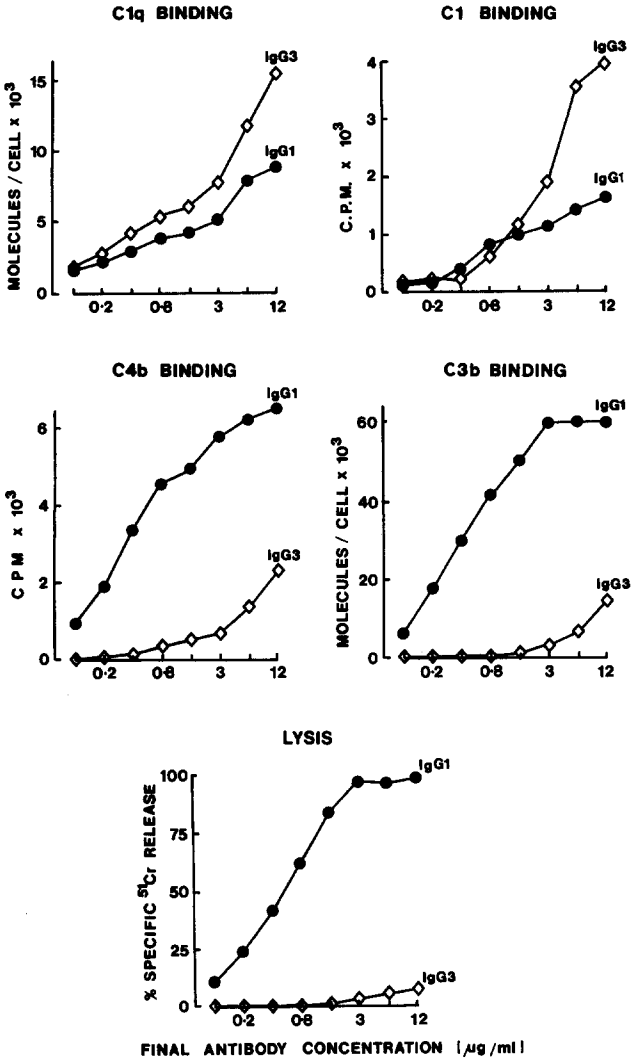

FIGURE 5. Dependence of C1q binding, C1 binding, C4b binding, C3b binding, and lysis on concentration of the sensitizing antibody. NIP-RBC were sensitized with IgG1 (●) or IgG.G3m(g) (◇) antibodies at a range of concentrations. Complement activation and cell lysis were measured as before. When the specific activity of the radiolabeled reagents was known, the results were calculated in terms of molecules bound per cell, otherwise they were recorded as measured cpm.

antibody was eluted from the cell surface under mild conditions using soluble hapten. The efficacy of this procedure was demonstrated using ^{125}I-labeled IgG1 or IgG.G3m(g) bound to NIP-RBC. To one sample, NIP-caproic acid was added, and this was compared with a control sample of antibody-coated NIP-RBC to which no free hapten was added. In the presence of added NIP-caproic acid only 3–6% of the antibody remained bound to the cell surface, while in the control samples, >90% still remained bound after 45 min (Table II). Elution of antibody occurred equally well when cells had been incubated in C6-deficient serum to allow activation of complement components C1 to C5 (data not shown).

Using this elution technique we tested whether removal of the antibody also caused removal of bound C4b and C3b, or whether they remained attached to the red cells.

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TABLE II
Elution of Anti-NIP Antibodies from the Cell Surface by NIP-Caproic Acid

Isotype of labeled anti-NIP antibody	NIP-caproic acid (0.1 mM)	^{125}I-labeled antibody remaining bound to the cell surface			
		5 min	20 min	40 min	80 min
		%			
IgG1	+	5	6	5	4
IgG1	–	94	90	94	86
IgG.G3m(g)	+	3	4	4	4
IgG.G3m(g)	–	95	94	96	92

NIP-RBC were labeled with ^{125}I-anti-NIP antibodies then incubated with or without NIP-caproic acid. The percentage of radioactivity that remained cell bound was measured after the cells had been separated from released antibody by centrifugation through oil.

The IgG1 and IgG3 anti-NIP antibodies were incubated with NIP-RBC in the presence of C6-deficient serum, and deposition of C4b or C3b was assayed with ^{125}I-anti-C4 or ^{125}I-anti-C3 as before. Addition of NIP-caproic acid had very little effect on the bound anti-C4 and anti-C3 antibody (Table III). This suggests that most, if not all, of the C4b and C3b were fixed on the cell surface rather than on the antibody molecule, for both IgG1 and IgG3 antibodies.

C4 Activation. Having shown that C4b was not efficiently deposited on the membranes when cells were sensitized with IgG3, we measured the extent of C4 activation in the presence of IgG1 or IgG3. To do this we assayed residual C4 activity in the supernatant and in order to obtain a measurable decrease it was necessary to use a higher cell concentration than in the previous experiments. The results (Fig. 6) showed that the rate of C4 activation with IgG3 was much less than that seen with IgG1, showing that the poor lytic activity of IgG3 was likely to be due to inefficient activation of C4 rather than inefficient deposition of activated C4 on the cell membrane.

TABLE III
Effect of NIP-Caproic Acid on C4b and C3b Binding

Antibody isotype	NIP-caproic acid added			Control		
	Bound cpm	Free cpm	Percent bound	Bound cpm	Free cpm	Percent bound
C4b binding						
IgG1	22,000	770	97	22,800	720	97
IgG.G3m(b)	11,700	650	95	12,200	540	96
IgG.G3m(g)	7,400	380	95	7,800	350	96
C3b binding						
IgG1	9,000	220	98	9,700	210	98
IgG.G3m(b)	2,500	210	92	2,900	140	96
IgG.G3m(g)	1,500	130	92	1,600	50	97

NIP-RBC sensitized with IgG1 or IgG3 anti-NIP antibodies were incubated with C6-deficient serum then washed and incubated with ^{125}I-anti-C4 or ^{125}I-anti-C3. The cells were washed again and incubated with or without NIP-caproic acid to elute the sensitizing antibody. The percentage of C4b or C3b attached to the cells was calculated from the bound and free radioactivity measured after centrifuging the cells through oil.

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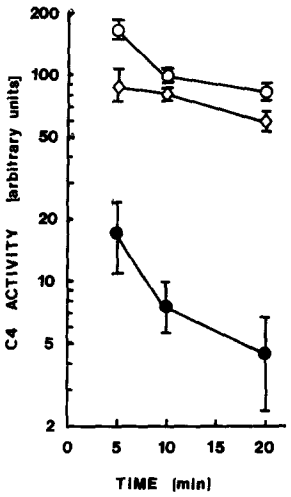

FIGURE 6. Rate of activation of C4. NIP-RBC were sensitized with IgG1 (●) or IgG.G3m(g) (◇) antibodies or medium alone (○) and incubated with C6-deficient serum for various times. Samples of the supernatant were assayed for residual C4 activity by titration in a hemolytic assay.

Discussion

In view of current efforts to obtain "humanized" antibodies for therapy, it is important to establish the range of effector functions of the individual human isotypes. Ideally, one would like to optimize all available effector mechanisms for clearance of cells in vivo. Different studies have demonstrated the importance both of cellular mechanisms (32, 33) and of complement fixation (34, 35). Our observations that the IgG1 isotype is the most effective for both complement lysis and antibody-dependent cell-mediated killing (9) suggest that this subclass is likely to be the one of choice.

The superiority of IgG1 for lysis was demonstrated with both human and with guinea pig complement, although the absolute levels of lysis were greater with the heterologous complement. Complement that is homologous with respect to the target cells is almost invariably less efficient for lysis (21, 22), due to cell membrane factors such as decay-accelerating factor and the homologous restriction factor which inhibit complement activation in a species-restricted manner (23–26). However, the fully homologous system is the only one relevant to therapy, so in the studies of individual stages of the complement cascade, we used human components and sera.

When the efficiency for lysis was measured by antibody titer, the hierarchy between isotypes (human IgG1 > rat IgG2b > human IgG3 > human IgG2 > human IgG4 > human IgE) was identical for different complement sources. As the various isotypes had the same V region and presumably identical binding affinity, the same number of antibody molecules should have been bound at any particular input concentration. Therefore, a higher antibody titer implies that fewer antibody molecules bound per cell are required to give lysis. Conditions that reduce the sensitivity of the target cells to lysis will require a higher antibody concentration to give the same amount of lysis. This was illustrated by the comparison of homologous complement (Fig. 1 *a*) with heterologous complement (Fig. 1 *b*). In a therapeutic situation we might expect further reductions in the sensitivity of the cells to lysis, first because the target antigen may be at lower density, and second, because nucleated cells require more complement channels for lysis. Therefore, the 100–500-fold difference

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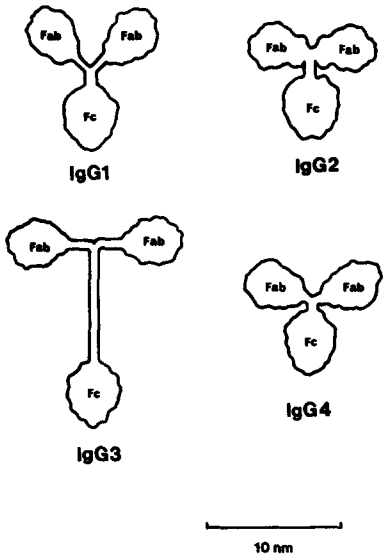

FIGURE 7. Structure of the human IgG isotypes, redrawn from reference 52 with permission.

in titers between the IgG1 and IgG3 antibodies will be critical and possibly only the IgG1 antibody will give any lysis at all.

To investigate why IgG1 was so lytic we studied various stages of the complement activation sequence. C1q binding was only detected with IgG3, IgG1, and rat IgG2b antibodies, but not with IgG2, IgG4, or IgE antibodies. The hierarchy agreed well with previous results for human myeloma proteins. Affinity constants for binding of C1q to monomeric human IgG in solution are as follows: IgG3 (K_a = 2.9 × 10^4/M), IgG1 (K_a = 1.2 × 10^4/M), IgG2 (K_a = 0.64 × 10^4/M), IgG4 (K_a = 0.44 × 10^4/M) (8). We failed to detect C1q binding by IgG2 and IgG4 antibodies, but it is likely that some binding that was too weak to detect in the assay used did take place, which would account for the hemolysis obtained with guinea pig complement.

Scatchard analysis showed that the IgG.G3m(g), IgG.G3m(b), IgG1, and rat IgG2b antibodies all had similar functional affinity constants for C1q binding of ~1.5 × 10^8/M. This was comparable with previous estimates of functional affinity constants for bivalent binding of C1q (36), and as expected, several orders of magnitude higher than the values for monomeric binding. Differences were observed in the numbers of C1q binding sites per cell; for example, the IgG.G3m(g) appeared to provide ~1.35–1.7 times as many binding sites as the IgG1 antibody. Despite considerable information indicating differences in the C1q binding ability of various isotypes in the case of the human (8), mouse (37), and rat (38, 39), the reasons for these differences are still not entirely clear. C1q binds to the CH2 domain of the antibody Fc region, and three different sites have been proposed (40–42). However, the primary sequence in each is very similar between the various isotypes. Some have suggested that differences between isotypes arise due to the flexibility of the antibody molecule, and depend on the length of the hinge region (43–44). Human IgG3 has an extraordinarily long hinge compared with the other isotypes (Fig. 7). This could result in more C1q binding sites on the cell surface as there would be more opportunities for two antibody Fc regions to come into appropriate proximity.

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Previous observations have suggested that C1q and C1 binding may be independently regulated (45) and that bound C1 is not necessarily activated (19, 46). It was therefore possible that a difference at this level might explain the paradox between C1q binding and hemolysis. In fact, the superiority of the IgG3 antibodies in C1q binding was even more marked when the binding of whole C1 was studied (Fig. 3).

When the binding of C4 and C3 were measured, a very different picture was obtained (Table I, Fig. 5) and now the isotype hierarchy was the same as in hemolysis. This suggested that the critical difference between IgG1 and IgG3 occurred between the stages of activated C1 and the fixation of C4 on the cell surface. After the enzymatic cleavage of C4 and C3, an activated molecule is produced, where the internal thiolester has the capacity to form a covalent bond with an acceptor nucleophile (hydroxyl or amino group) on the cell membrane. However, the activated molecule has a half-life of only about 25–30 μs in solution (47, 48) because the thiolester is rapidly hydrolyzed. Furthermore, not all hydroxyl or amino groups are equally efficient as acceptor molecules (49) and only a fraction of C4b and C3b is deposited at the complement activation site. The proportion that binds will depend on the precise nature and vicinity of acceptor groups that are available. One hypothesis to explain why IgG1 gave more C4b binding was that the IgG1 constant region might have been a particularly good acceptor. Studies using immune complexes or antibody-coated bacteria have shown that antibody can be a good acceptor of both C4b (28) and C3b (29–31). In contrast, experiments using red cells coupled with methotrexate suggested that both C4b and C3b were bound to the cell surface rather than to the antibody molecule (50, 51). Our results are consistent with these latter findings. The ability of the chimeric antibodies to provide an acceptor site could not account for differences in C4 fixation, as most of the C4b and C3b were bound to the cell membrane (Table III).

The average distance that C4b can diffuse during the half-life of the nascent thiolester bond is ~6 nm, rather less than the relaxed length of the hinge region (9 nm) in IgG3 (Fig. 7) (52). Therefore, it was conceivable that C4 was activated but did not reach the cell surface before decaying. This explanation was ruled out by measurement of residual (unactivated) C4 in the cell supernatants (Fig. 6). It appeared that C1s activated via IgG3 was very inefficient at activation of C4.

Perhaps the unusual size of the IgG3 molecule may allow access of C1 inhibitor to the activated C1; C1 inhibitor is a large serum protein, which can usually only bind to C1 in solution. Thus, C1 inhibitor may attach to the activated C1s and block C4 activation. We did not see the C1-inhibitor-C1r complex at ~135 kD on the SDS gel, but C1-inhibitor-C1s would have been invisible because the 27-kD fragment of C1s does not contain an iodination site. The other possibility is that binding of C4 to the C1 complex is favored by IgG1 but not by IgG3. We could imagine a noncovalent binding site for C4 on IgG1, but not on IgG3, which stabilizes the interaction. Alternatively, perhaps the long flexible hinge of IgG3 allows proportionally more three-headed binding of C1, preventing C4 from gaining access to the active site. These theories could be distinguished by using chimeric antibodies with appropriate genetic changes.

So far these results hold for the model system of hapten-coated erythrocytes. This was convenient for our experiments but not of great physiological importance. However, recent studies with an analogous matched set of chimeric human mAbs specific

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for the human lymphocyte antigen CAMPATH-1 have shown the same paradoxical hierarchy of complement lysis when lymphocytes are used as targets (53), and so IgG1 does seem to be superior for cell lysis in general. In different circumstances it is possible that IgG3 has a more important role in complement fixation, for example in clearance of immune aggregates or of bacteria or viruses. At present we cannot say whether the poor C4 fixing ability would apply in those situations.

An important conclusion from the work presented here is that antibody isotype can influence complement-activating function at a level other than the binding of C1q, namely at the level of C4 activation. Future efforts to improve the lytic ability of mAbs by genetic engineering will need to allow for the effect of antibody structure on this stage in the complement cascade. By appropriate genetic manipulation it might be possible to combine the greater C1-activating ability of the IgG3 with the greater C4 fixing capacity of IgG1 to produce an antibody with even better lytic capability than any of the native isotypes.

Summary

Humanized antibodies are likely to have a major role in therapy and it is important to define their interaction with physiological effectors. By comparing a matched series of chimeric human mAbs we found that IgG1 was most efficient in complement lysis, although IgG3 bound more C1q. To resolve this paradox we compared the ability of human IgG1, IgG2, IgG3, IgG4, and IgE and rat IgG2b to cause C1q binding, C1 binding and activation, C4 activation, C4b binding, and C3b binding. Rat IgG2b was included because this isotype has already successfully been used for therapy. Human IgG1 was less efficient than IgG3 and fixing C1q and C1 on the cell surface, but the number of C4 molecules bound per C1 was 10-fold greater for IgG1 than for IgG3. This difference, amplified through later stages of the complement cascade, can account for the superiority of IgG1 for cell lysis. The efficiency of IgG1 in fixing C4 was not due to a favored binding site on the antibody molecule, since virtually all of the bound C4b was attached to the cells. Rather, it appeared that the activation of C4 by C1s was greatly favored by IgG1 compared with IgG3. It should be possible to combine the optimal properties of IgG1 and IgG3 antibodies to produce an improved therapeutic reagent.

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