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Activation of complement by human IgG1 and human IgG3 antibodies against the human leucocyte antigen CD52

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SUMMARY

Activation of the complement cascade by immunoglobulin G (IgG) plays a major role in the host defence against pathogens. Using recombinant human antibodies specific for the leucocyte antigen CD52, different allotypes of human IgG1 subclass were compared for their ability to activate human complement. In addition the roles of the different length hinge regions of IgG1 and IgG3 were investigated. It was found that the naturally occurring allotypes G1m(a,z) and G1m(f), and one artificially created isoallotype, G1m(null), did not significantly differ in their overall ability to cause cell lysis. However, some differences in binding of individual components of the classical activation pathway were detected. More of the complement component C1s seemed to be associated with the allotype G1m(f), although this did not result in an overall improvement in lytic potency. In this system the wild-type IgG3 was found to be less effective in complement lysis than IgG1. By shortening the hinge region of IgG3 to resemble that of an IgG1 antibody, increased complement binding was observed compared with that of wild-type IgG3 and the IgG1 allotypes. The overall lytic potency of the antibody was also improved compared with wild type IgG3 and it was also slightly more effective than the IgG1 allotypes.

INTRODUCTION

The four subclasses of human immunoglobulin G (IgG), (IgG1–4), differ in their abilities to mediate effector functions. Hierarchies of complement activation have indicated that IgG1 and IgG3 are the most potent subclasses at mediating activation of the complement cascade, whilst IgG2 and IgG4 are very poor activators.[1] Within both the IgG1 and IgG3 subclasses, further polymorphisms exist in the constant domains of the IgG heavy chains, termed allotypes (allotypes of IgG subclasses are denoted by Gm).[2,3] The role of the allotypes in effector functions is unclear, and has been little studied. In this work, human IgG1 allotypes with specificity to the human lymphocyte surface antigen CD52 were investigated for their abilities to activate the human complement cascade. Three IgG1 allotypes were studied, which differ in residues present in the C_H1 and C_H3 domains. The allotype G1m(a,z) has the allotypic residues LYS 214 in the C_H1 domain, and ASP 356/LEU 358 in the C_H3 domain.[4,5] The allotype G1m(f) has ARG 214 in the C_H1 domain, and GLU 356/MET 358 in the C_H3.[6] The novel isoallotype G1m(null) has THR 214 in the C_H1 domain, and GLU 356/MET 358 in the C_H3.[7] This novel allotype was constructed to produce an antibody which did not react with the human IgG1 anti-allotype antisera.[7]

Previous studies have incorporated the use of rodent or human antibodies, or chimeric rodent–human antibodies directed against a variety of hapten antigens, to study the influence of subclass on activation of heterologous complement, usually of guinea-pig or rabbit origin.[8] By using heterologous serum, and heterologous cells, the activation of complement is enhanced by the absence of appropriate homologous restriction factors on the surface of the target cell which otherwise protect host cells from the effects of complement activation. The work described here uses an entirely homologous system involving human antibodies against a human antigen and human complement activation.

The role of the different hinge regions in IgG1 and IgG3 in the activation of the human complement cascade was studied. IgG1 has a hinge region of 15 amino acids, whilst the hinge of IgG3 is much larger, being 62 amino acids in size. The hinge of IgG3 has the highest degree of flexibility amongst the IgG subclasses.[9,10] A mutation in the hinge region of IgG3 was made by removing the first three hinge exons, and leaving a hinge comprised 15 amino acids and all of the lower hinge. This type of mutated IgG3 antibody has previously been reported as having improved capacity for complement

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mediated lysis.[11] The structure of the hinge regions of the IgG3 wild type and deletion mutant antibodies is shown in Fig. 1. The work described here makes use of an entirely homologous system involving human antibodies against the human leucocyte antigen CD52. Several antibodies to this target antigen have been used therapeutically and a humanised IgG1 antibody called CAMPATH-1H was selected partly based on it's ability to lyse human lymphocytes effectively in the presence of autologous complement.[12]

The complement system comprises both serum and cell surface proteins. Activation of the system initiates a sequence where each component activates the next in a cascade fashion, allowing considerable amplification to occur. In this work, the activation of the classical pathway was studied. The classical activation pathway consists of components C1, C2, C3 and C4. Activation of this pathway results in completion of the terminal pathway, with resultant lysis of target cells.

MATERIALS AND METHODS

Production of human IgG1 and IgG3 anti-CD52 monoclonal antibodies

Hybridomas expressing the anti-CD52 human IgG1 antibodies, and having allotypes G1m(a,z), G1m(f) or G1m(null) have been previously described.[12,13] Cell lines were cultured in Iscove's modification of Dulbecco's medium (Gibco, Paisley, UK) in 2% γ-globulin-free bovine fetal calf serum (Gibco). IgG1 was purified directly from spent tissue culture supernatant onto Protein A–Sepharose Fastflow columns, and IgG3 on Protein G–Sepharose (Sigma, Poole, UK). The antibodies were eluted with 100 mM glycine at pH 3·0 and into pH 7·0 buffer to neutralise, before dialysis against phosphate-buffered saline (PBS).

Stable transfectants expressing anti-CD52 human IgG3 and the hinge deletion mutant IgG3 (m15 IgG3) were created using the rat myeloma cell line Y0. 40 μg of linearized heavy chain vector and 80 μg of light chain vector were transfected by electroporation using a Gene Pulser (BioRad, Hemel Hempstead, UK) at 0·25 kV × 200 Ω. Transfectants were selected with mycophenolic acid (5 mg/ml) and xanthine (5 mg/ml in 5 M NaOH). Positive colonies were cloned by limiting dilution, and expression of antibodies was detected in culture supernatants by enzyme-linked immunosorbent assay (ELISA) using anti-human IgG Fc (Sigma) as capture antigen and biotinylated anti-human κ (Sigma) as detection reagent. Antibodies were purified from culture supernatant as described.

Antibodies were examined on sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing and non-reducing conditions using a Phastsystem separation and control unit (Pharmacia LKB, Upsaala, Sweden). Concentration of antibodies was estimated by competitive binding assay.[14] An ELISA was used to confirm the allotype of the purified antibodies. Plates were coated with anti-allotype monoclonal antibodies (Netherlands BTS, Amsterdam, the Netherlands), and bound proteins were then detected with biotinylated anti-human κ light chain monoclonal antibody (Sigma) (Fig. 2).

IgG3

upper hinge	middle hinge	lower hinge
ELKTPLGDTTHT	CPRCP	APELLGGP
	(EPKSCDTPPPCPRCP)X3	

IgG3 hinge deletion mutant

upper hinge	middle hinge	lower hinge
absent	EPKSCDTPPPCPRCP	APELLGGP

Figure 1. The structure of the hinge regions in the IgG3 wild-type and hinge deletion mutant antibodies.

Complement-mediated lysis assay

Peripheral venous blood taken from healthy volunteers (60 ml) was defibrinated, and peripheral blood mononuclear cells (PBMC) were recovered using Nycoprep density gradient separation (Nycoprep, Nycomed, Norway). Human serum to be used as a source of autologous human complement was removed from the same preparations. PBMC ($2–5 \times 10^7$/ml) were labelled with ^{51}Cr for 1 hr at 37°. The cells were then transferred to a 96-well plate (100 μl of cells at 1×10^5/well) and incubated with antibody in the presence of autologous serum for 1 hr at 37°. Supernatant was then harvested and counted using a Wallac automatic γ counter (Pharmacia).

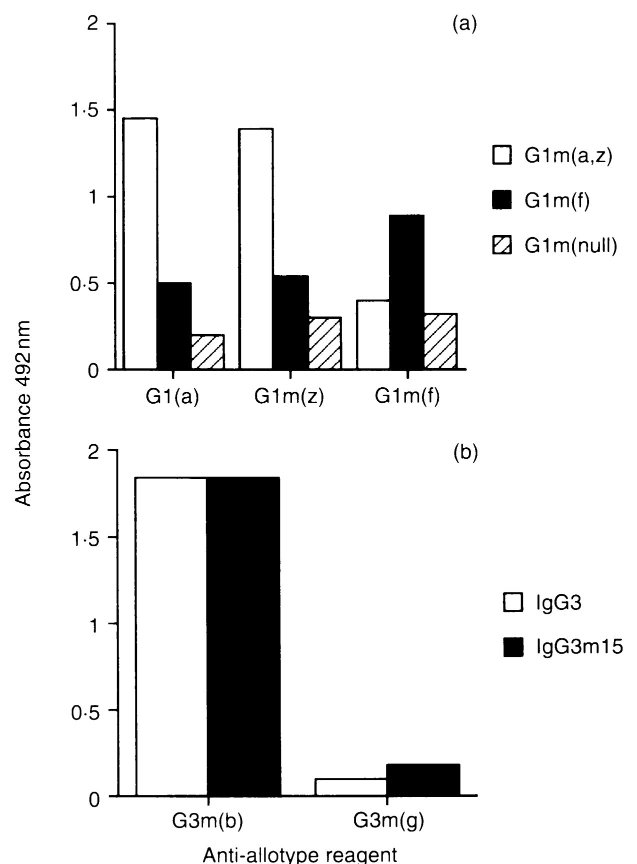

Figure 2. Determination of the allotype of each of the IgG1 and IgG3 antibodies by ELISA. Purified antibodies from hybridoma supernatant were captured on 96-well plates using anti-allotype monoclonals, anti G1m(a), anti-G1m(f), anti-G1m(z), anti-G3m(b) or G3m(g) (Netherlands BYS, Amsterdam, Holland) at 50 μg/ml. Bound antibodies were detected with biotinylated anti-human κ light chain monoclonal antibody (Sigma) and streptavidin HRP (Sigma).

Percentage of ^{51}Cr release, related to cell lysis, was determined by calculating

$$\%\text{specific release} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum counts} - \text{spontaneous release})} \times 100$$

where spontaneous release was cpm of cells incubated without antibody or serum, and maximum counts was the c.p.m. of 100 µl of re-suspended target cells.

Complement component binding assay using C9-depleted serum
Human PBMC were prepared as before, and re-suspended at 1×10^4/well in a 96-well plate. Cells were incubated with antibody for 1 hr at 4°, followed by incubation either with autologous human serum or C9-depleted human complement (The Binding Site, Nottingham, UK) for 1 hr at 37°. The cells were washed and then stained with anti-complement component fluorosceinisothiocyanate (FITC)-conjugated monoclonal antibodies against C1q, C1s, C4 and C3 (The Binding Site) for analysis by flow cytometry using a FACScan (Becton Dickinson, Mountain View, CA).

RESULTS

Complement mediated lysis by human IgG1 allotypes

The human IgG1 allotypes G1m(a,z), G1m(f) and an isoallotype G1m(null) were compared for their relative abilities to produce complement mediated lysis against human PBMC target cells, using autologous human serum as a source of complement. There was overall little difference between the three antibodies in terms of percentage cell lysis observed (Fig. 3). At concentrations of 6–50 µg/ml, the antibodies were able to mediate between 60–84% lysis. At concentrations between 0·75–3 µg/ml, the antibodies were able to produce between 5–20% cell lysis.

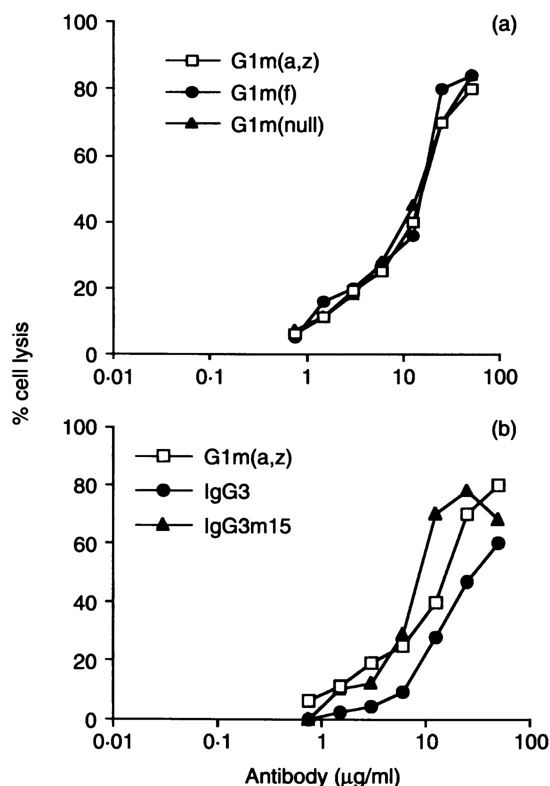

Figure 3. Complement mediated lysis of human cells by human IgG1 and IgG3. (a) Human PBMC (1×10^6/well), labeled with ^{51}sodium chromium (Amersham), were incubated with the allotypes of human IgG1 G1m(a,z), G1m(f) and G1m(null) at concentrations of up to 50 µg/ml. Autologous serum, as a source of complement, used at a dilution of 1/4 in IMDM was added, and the assay was incubated for 1 hr at 37°, before harvesting of the culture supernatant to determine ^{51}Cr release, to determine cell lysis. Spontaneous release was always less than 5% of maximum c.p.m. Maximum c.p.m. typically was between 3000–5000. The data shown is representative of three independent experiments. (b) Assay as for above, using human IgG3 wild type or human IgG3 hinge deletion mutant (m15 IgG3).

Complement mediated lysis by human IgG3 and a hinge deletion mutant of IgG3

Two anti-CD52 antibodies of IgG3 subclass, one with a deletion in the hinge region reducing the hinge in size from 62 amino acids to 15 were compared for the ability to produce complement-mediated lysis. It was found that the m15 IgG3 of the IgG3 was more effective at complement activation than the wild type IgG3 antibody. At 12–25 µg/ml, the hinge deletion mutant IgG3 was able to produce 65–70% cell lysis, whilst the wild type IgG3 produced 28–42% cell lysis. The m15 IgG3 was comparable to the IgG1 allotype G1m(a,z) at mediating complement lysis at concentrations ranging from 0·75–50 µg/ml of antibody.

Binding and activation of complement components of the classic pathway by IgG1 allotypes

This assay was used to compare the abilities of the human G1m allotypes in activating the classic human complement pathway. The assay measures the deposition of complement components associated with the cell surface. No significant difference was observed in the abilities of G1m(a,z), G1m(f) and G1m(null) to bind C1q (Fig. 4). Interestingly, however, there did appear to be differences in the degree of activation of C1s (part of the $C1rC1s)_2$ complex) amongst the allotypes. The allotype G1m(f) was able to activate C1s slightly more efficiently than either G1m(a,z) or G1m(null). The next component of the complement cascade is C4, which is cleaved by C1s, however the allotypes of IgG1 did not show any marked differences in their ability to activate C4, despite the apparent increase in deposition of C1s afforded by the G1m(f) allotype. There was a small reduction in detection of C3 with the G1m(f) allotype, as compared to the other two antibodies tested.

Binding and activation of complement components of the classic pathway by IgG3 wild-type and hinge deletion mutant antibodies

In contrast to the IgG1 allotypes, there were marked differences in the abilities of the two IgG3 antibodies to bind to, or activate each component of the classical complement pathway. Using the m15 IgG3, more C1q, C1s, C4 and C3 was detected at the cell surface than when the wild-type IgG3 was used. The m15 IgG3 was able to bind to C1q, and activate C1s, C4

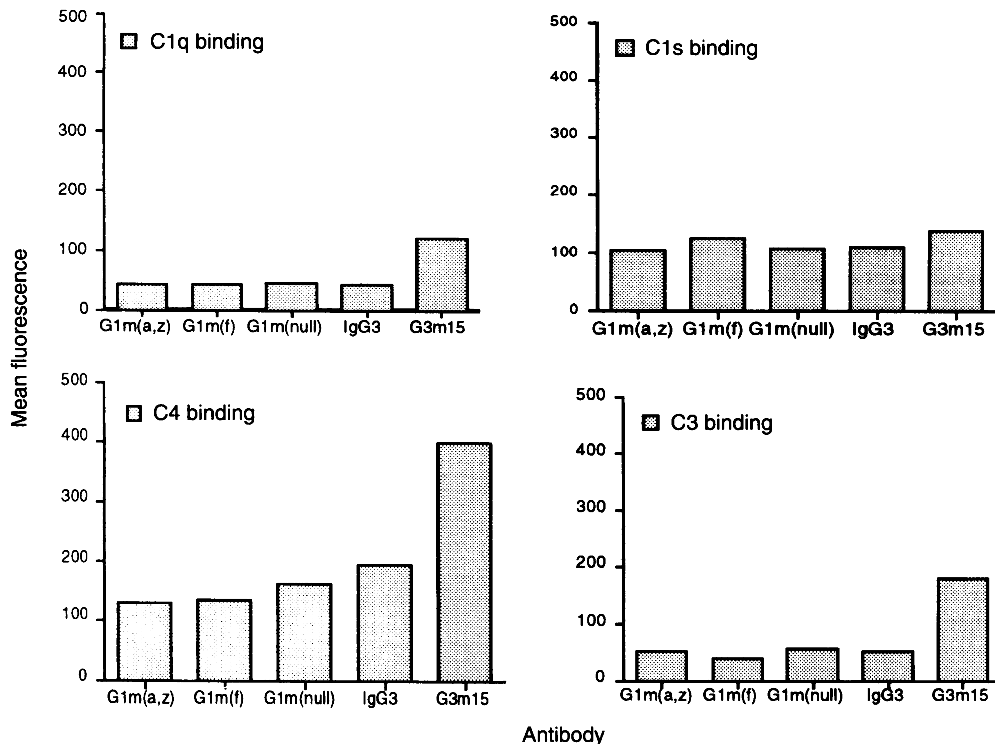

Figure 4. Binding of complement components C1q, C1s, C4 and C3 by human IgG antibodies. Human PBMC (5×10^4/well) were incubated with allotypes of human IgG1 (G1m (a,z), G1m(f) or G1m(null)) or with two IgG3 antibodies (IgG3 wild-type and a hinge deletion mutant m15IgG3) with specificity against human CD52. Antibodies were at a concentration of 25 μg/ml. Cells were incubated with antibody and with C9-depleted human serum (The Binding Site) for 1 hr at 37°. Detection of complement components bound to the cell surface was then made using FITC-conjugated monoclonals against C1q, C1s, C4 and C3 (The Binding Site). Cells were fixed in 4% paraformaldehyde before analysis by flow cytometry on a fluorescence-activated cell sorter (FACScan; Becton Dickinson, CA). Mean cell fluorescence for 20 000 events was determined. Control samples of unstained cells, or secondary antibody alone had a mean cell fluorescence of 10–20. The data shown is representative of three independent experiments.

and C3 to a greater extent in each case, than was the wild type IgG3. The m15 IgG3 was improved in its binding to C1q (three times greater), activation of C1s (0·5 times greater), of C4 (two times greater) and of C3 (3·5 times greater), compared to the wild-type IgG3. Additionally, detection of C1s, C4 and C3 was greater using the m15 IgG3 than with all three of the IgG1 allotypes tested.

DISCUSSION

The aim of this work was to compare allotypes of human IgG1, as well as IgG3 wild type and a hinge-shortened mutant thereof (m15), anti-human CD52 antibodies for their ability to activate the human complement cascade. All antibodies were identical in the antigen binding region. Comparisons were made in terms of overall lytic potency, and the ability of each antibody to bind to or activate components of the classical activation pathway, C1q, C1s, C3 and C4.

In therapeutic situations antibodies against human cells are generally poorly lytic with human complement because of homologous restriction factors present on the cells. A heterologous source of complement is almost always superior to autologous complement for lysis, and this needs to be considered when interpreting *in vitro* assays for antibody effector function. In work described here, human antibodies were used in a system with autologous complement and human cells.

There was little difference observed in overall lysis produced by the three allotypes of human IgG1. The allotypic residues are present in the C_H1 and C_H3 domains of the antibodies, whilst the binding site for C1q, the initiator of the classic activation pathway, is believed to be in the C_H2 domain. In mouse IgG2b, the binding motif for C1q has been reported as Glu318–Lys 320–Lys 322.[15] However, all the subclasses of human IgG have this motif, so clearly there are other determinants involved in complement activation because the subclasses differ in their lytic potential. Roles for residues in the C_H1 and C_H3 domains in complement activation have been suggested, and a possibility that allotypes might differ in their ability to activate complement was investigated here. It was found, however, in this assay system that the allotypes of IgG1, which have polymorphisms in their C_H1 and C_H3 domains, were equivalent in their overall ability to produce complement-mediated lysis (CML), and therefore, it would appear that these allotypic residues do not exert a distinguishing effect on this particular effector function of the antibodies.

A comparison of the two IgG3 antibodies revealed that a hinge deletion mutant of IgG3 (m15) was more efficient in CML than the wild-type IgG3. For the CD52 antigen system

it had previously been found that the IgG1 isotype was better in CML than the IgG3 isotype, and so a humanised IgG1 of allotype G1m(a,z) was chosen for further therapeutic development.[12] This previous study had made use of chimeric antibodies with a rat κ light chain and a rat V_H expressed along with human heavy chain constant regions.[12] We have confirmed here, using fully humanized heavy and light chain CD52 antibodies, that the lytic titre of IgG1 is higher than IgG3. However, we have also shown that an IgG3 mutant with a similar length hinge to IgG1 gives improved lysis and may have slightly higher lytic potential. Using chimaeric antibodies to hapten antigens and heterologous target and complement it has previously been suggested that IgG3 is better than IgG1 but that this hierarchy was dependent on the hapten density and at higher densities IgG1 was found to be sometimes better than IgG3.[16,21] In this CD52 antigen system we have found that IgG1 is more potent and is thus more similar to the results obtained at higher hapten density, and we can also confirm that it is the longer hinge of IgG3 compared to IgG1 which seems to reduce the lytic potency of this isotype.

The ability of each of the IgG1 allotypes and the IgG3 antibodies to bind to or activate the components of the classic activation pathway was also investigated. The first stage in the classic activation of complement is the binding of antibody to the C1 complex. The C1 complex comprises C1q and $(C1rC1s)_2$.[18] Binding of C1q is the initial step and can occur in the absence of C1r and C1s. Binding of C1q to each of the IgG1 allotypes, and to the wild-type IgG3 was found to be relatively low. Previous work had indicated that IgG3 was more effective at binding to C1q than was IgG1.[19] However, the m15 IgG3 was the most effective of the antibodies tested at binding C1q, with an approximately fourfold increase in the level of binding compared to wild type IgG3. It is possible to speculate that by shortening the hinge region of IgG3, and bringing the C_H1 and C_H2 domains closer together there is improved binding and stability of the complex formed when C1q contacts the antibody.

C1s, part of $(C1rC1s)_2$, is a serine esterase which is activated by the binding of C1q. Activated C1s can then bind to and activate C4, the next stage in the cascade. This is the major amplification step in the cascade, with obvious importance for this reason. There were differences observed amongst the allotypes in the activation of the C1s component of the next step in the cascade. The G1m(f) allotype, which has ARG 214 as the allotypic residue in the C_H1 domain, was the most efficient of the IgG1 allotypes at binding of C1s, and of further interest is that the m15 IgG3 also has an ARG at this equivalent position. Again, this may indicate a contribution from the C_H1 domain to the binding site for the C1 complex of the complement cascade.

A structural model of the C1 complex has been proposed, in which the $(C1rC1s)_2$ tetramer is interwoven between the arms of the C1q molecule.[20] It is generally accepted that C1q associates with C1r and C1s in a 1:2:2 ratio. However, it is not possible to interpret the findings presented here by discussing precise stoichiometry, because here, detection of the components was being made in an indirect assay. Hence, the precise number of molecules of C1q and C1s on the cell surface could not be measured, as is possible using, for example, directly labelled components. The binding of the FITC–IgG anti-complement component antibodies is likely to be dependent not only upon the amount of complement component on the cell surface, but also on it's steric availability. When comparing results obtained with the antibody against C1q with the anti-C1s antibody, it cannot be assumed that these would give a similar dose–response curve for absolute quantitation. It may be concluded from the assay used here that the different allotypes of IgG1, and the two IgG3 antibodies, which were used to activate the complement cascade, differing amounts of complement components accessible on the cell surface were detected when probed with antibodies specific for the individual complement components.

Detection of C1s was higher with the m15 IgG3 antibody than with any of the IgG1 allotypes, and also the IgG3 wild type antibody. This correlated with the increased level of binding to C1q, and the overall improvement of lytic ability. Interestingly IgG3 has an ARG as the equivalent residue to the allotypic position 214 in IgG1 and the G1m(f) allotype (ARG 214) was the most efficient at activation of C1s of the three IgG1 allotypes tested.

The next stage in the complement cascade, following C1 complex activation, is the cleavage of C4. One of the cleavage products, C4b anchors the next component, C2, to the cell surface, forming a complex of C4bC2 which will cleave C3 – the central step in the pathway. In the assay system used here, the majority of the C3 and C4 activation was measured by deposition on the cell surface of the activated components C3b and C4b. There may also be some interaction between these components and the C_H1 domains of the antibodies. There was no significant difference in the activation of either C4 or C3 by each of the IgG1 allotypes detected in this assay, despite the differences seen in the binding to C1s. The differences in the C_H1 domains of the allotypes are not reflected by a capacity to induce more efficient activation of the C3 or C4 components. It would also appear that, despite the improvement in binding to C1s by the G1m(f) allotype, overall it was not sufficient to lead to an increase in complement activation by this antibody. The IgG3 wild-type antibody was able to activate C4 to the same level as the IgG1 allotypes, whilst the m15 IgG3 was better at activation of both C4 and C3 than any of the other antibodies. Previous reports have indicated that IgG3 was more effective at activation of C4.[19] The potency of the m15 IgG3 at binding each component of the pathway agrees with the overall improvement in the antibody's ability to produce CML.

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