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Structural Difference in the Complement Activation Site of Human IgG1 and IgG3

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Abstract

The C1q binding epicentre on IgG molecules involves residues Asp^{270}, Lys^{322}, Pro^{329} and Pro^{331} in the C_H2 domain. IgG1 and IgG3 are usually the most efficient of the four human IgG subclasses in activating complement and they both share all these residues. To reveal possible differences in the structural requirement for complement activation, we created a number of NIP (5-iodo-4-hydroxy-3-nitro-phenacetyl) specific IgG1 and IgG3 antibodies with parallel mutations in or near the putative C1q binding site. The mutants were tested simultaneously for antibody induced, antibody-dependent complement-mediated lysis (ADCML) at high and low antigen concentration on the target cells using sera of human, rabbit and guinea pig as complement source. In addition, we tested the antibodies against target cells decorated with the NP hapten, which has 10-fold lower affinity for the antibodies compared to the NIP hapten. We also used ELISA methods to measure complement activation. We observed a clear difference between IgG1 and IgG3 localized to residues Asp^{270}, Leu^{334}, Leu^{335}. For all these residues, and especially for Asp^{270}, IgG1 was heavily reduced in complement activation, while IgG3 was only moderated reduced, by alanine substitution. This difference was independent of the long hinge region of IgG3, demonstrated by hinge region truncation of this isotype such that it resembles that of IgG1. This report indicates the presence of structural differences between human IgG1 and IgG3 in the C1q binding site, and points to a specialization of the two isotypes with respect to complement activation.

Introduction

Antibody-dependent complement-mediated lysis (ADCML) is initiated by the binding of two or more IgG to the surface of target cells, followed by a multivalent interaction between the IgG and two or more of the six globular heads of C1q [1]. It is generally accepted that recognition of IgG by C1q occurs via a site in the C_H2 domain [2–6]. Point mutations introduced in the IgG molecule demonstrate the importance, direct or indirect, of several residues, such as K322 [7, 8] and P329 and others [7]. Normally, IgG1 and IgG3 are the most efficient human isotypes for complement activation [9, 10], while IgG2 requires high antigen concentration in order to activate complement and IgG4 is negative [10, 11]. When the complement activation site of human IgG has been mapped by site-directed mutagenesis, IgG1 and IgG3 have been analysed in separate studies and directly comparable test systems have not been used. To compare IgG1 and IgG3 directly for possible structural differences in complement activation, we introduced the same site-directed mutations in both isotypes with shared NIP (5-iodo-4-hydroxy-3-nitro-phenacetyl) specificity, and tested them side by side for complement-mediated lysis against red blood cells labelled with high or low antigen loads, and also in ELISA based assays for complement activation. The results showed clear differences between IgG1 and IgG3 at residues L234, L235 and D270. When these residues were mutated to alanine, IgG1 was heavily reduced in complement activation, especially for the D270A mutation, while the parallel mutations in IgG3 molecule gave only moderate reduction in complement activation. The differences between IgG1 and IgG3 were independent of hinge length as shown by truncation of the long hinge region of IgG3 to resemble that of IgG1.

Materials and methods

Construction of mutant IgG1 and IgG3 antibodies. The constructed antibodies used in this study are listed in

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Table 1 Overview of the NIP-specific chimeric antibodies used in this study.

Name	Description
IgG1wt	IgG1 wild type
IgG1D270A	IgG1 where Asp at position 270 is mutated to Ala
IgG1L234A	IgG1 where Leu at position 234 is mutated to Ala
IgG1L235A	IgG1 where Leu at position 235 is mutated to Ala
IgG1K322A	IgG1 where Lys at position 322 is mutated to Ala
IgG1P329A	IgG1 where Pro at position 329 is mutated to Ala
IgG1P331G	IgG1 where Pro at position 331 is mutated to Gly
IgG3wt	IgG3 wild type
IgG3D270A	IgG3 where Asp at position 270 is mutated to Ala
IgG3L234A	IgG3 where Leu at position 234 is mutated to Ala
IgG3L235A	IgG3 where Leu at position 235 is mutated to Ala
IgG3K322A	IgG3 where Lys at position 322 is mutated to Ala
IgG3P329A	IgG3 where Pro at position 329 is mutated to Ala
IgG3P331G	IgG3 where Pro at position 331 is mutated to Gly
IgG3h1D270A	IgG3 with the hinge of IgG1 and where Asp at position 270 is mutated to Ala
IgG3h1K322A	IgG3 with the hinge of IgG1 and where Lys at position 322 is mutated to Ala

Table 1. All mutations were done by PCR guided site-directed mutagenesis involving primers specific for each mutation (Table 2). The template used was the shuttle vector pLNOH2 containing the constant part of either the human $\gamma 1$ or the $\gamma 3$ gene [12]. PCR conditions were 20 cycles with 30 s annealing at 56 °C, 4 min polymerization at 68 °C and 1 min denaturation at 96 °C, 10 ng of template, 20 pmol of each primer, 10 nmol nucleotides and 2 units of X-pand PCR polymerase in the appropriate buffer. The enzyme was added at 4 °C and the reaction was then brought rapidly to 96 °C before starting the PCR cycles. Five parallel reactions of 100 μl for each primer pair were conducted. The mutated DNA product was processed down streams by ethanol precipitation (Amersham SeeDNA kit; GE Life Science, Piscataway, NJ, USA), purified by Amersham SR400 MicroSpin columns, digested with *Sfi*I, and separated by agarose gel electrophoresis. The correct DNA fragments were ligated using T4 DNA ligase and introduced to *Hind*III/*Bam*HI site in the PLNOH2 vector. Correct insertion and structure of the gene was verified by restriction enzyme fragmentation pattern and gene sequencing.

The vector also contained an exon encoding a mouse variable heavy chain with specificity for the hapten NIP. These heavy chains can pair with the λ_1 light chains which were co-transfected into the 293E cell line as described [13]. Supernatants from transfected cells were quantified for presence of antibody by ELISA, in which microtitre plates were coated with 100 μl, 1 μg/ml $NIP_{16}BSA$ (BSA-conjugated with 16 NIP groups per molecule).

Quantitation of chimeric IgG in cell cultures. The amount of IgG, irrespectively of subclass, was quantitated by ELISA using microtitre plates as previously described [14]. Briefly, microtitre plates coated with $NIP_{16}BSA$ were reacted with dilutions (1–20 ng/ml) of a chimeric IgG3 standard stock solution, 1 mg/ml, stored at 4 °C (can be stored for 1 year), or dilutions of test samples. After incubation for 2 h at 37 °C, the plates were washed with PBS/T using a microplate washer (ScanWasher 300, Scatron, Lierbyen, Norway). Then the plates were incubated with an optimum mixture of locally produced, affinity purified, biotin-labelled sheep anti-human IgGFc (125 ng/ml), previously shown to react equally well with IgG1 and IgG3 [15], strepavidin (167 ng/ml) and biotin-labelled alkaline phosphatase (100 ng/ml). After incubation for 2 h at 37 °C, the plates were washed with PBS/T, the plates were developed by adding 100 μl

Table 2 Primers used to introduce the 234, 235, 270, 322, 329 and 331 mutations in IgG1 and IgG3 constant domains.

Name	Function	Sequence
D270A-1-IgG3	Used to introduce D270A mutation and *Bse*AI site in IgG3	5′-gccacgaa**GCT**ccggaggtccagttcaagtgg
D270A-2-IgG3	Used to introduce D270A mutation and *Bse*AI site in IgG3	5′-aactggacctccgg**AGC**ttcgtggctcacgtccacc
Reverse-IgG	Reverse primer at 3′-end of IgG containing *Bam*HI site	5′-actactactggatccgacccgctctgcctc
Forward-IgG	Forward primer at 5′-end of IgG containing *HindIII* site	5′-gtggtgatgaagctttctggggcaggccaggcctg
P329A/P331G-1	Used to introduce P329A/P331G double mutation and *Sfi*I site	5′-ggtctccaacaaggccctc**GCG**gcc**GGG**atcgagaaaaccatctc
P329A/P331G-2	Used to introduce P329A/P331G double mutation and *Sfi*I site	5′-tttctcgat**CCC**ggc**CGC**gagggccttgttggagaccttgcacttc
P329A-1	Used to introduce P329A mutation and *Sfi*I site	5′-ggtctccaacaaggccctc**GCG**gccccatcgagaaaaccatctc
P331G-1	Used to introduce P331G mutation and *Sfi*I site	5′-ggtctccaacaaggccctcccggcc**GGG**atcgagaaaaccatctc
P331G-2	Used to introduce P331G mutation and *Sfi*I site	5′-tttctcgat**CCC**ggcccgggagggccttgttggagaccttgcacttc
K322A-2	Used to introduce K322A mutation and containing *Bsa*I site	5′-gagggctttgttggagac**CCG**gcacttgtactccttgccgtt
D270A-1-IgG1	Used to introduce D270A mutation and *Bse*AI site in IgG1	5′-gccacgaa**GCT**ccggaggtccagttcaagtgg
D270A-2-IgG1	Used to introduce D270A mutation and *Bse*AI site in IgG1	5′-aactggacctccgg**AGC**ttcgtggctcacgtccacc
L234A-1	Used to introduce L234A mutation and *Bse*AI site	5′-Tcctcagctccggaa**GCC**ctgggaggaccgtcagtcttcctc
L235A-1	Used to introduce L235A mutation and *Bse*AI site	5′-tcctcagctccggaactc**GCC**cggaggaccgtcagtcttcctc
L234A,L235A-1	Used to introduce L234A,L235A double mutation and *Bse*AI site	5′-tcctcagctccggaa**GCCGCC**ggaggaccgtcagtcttcctc
Reverse 234,235-2	Reverse primer used to introduce L234A and L235 A *Bse*AI site	5′-cccaggagttccggagctgaggaagagatggagg
BglIIforwardLNO	Alternative forward primer used for amplification of IgG	5′-ggatcgggagatctcccgatcccctatg

Mutation site in capital bold, restriction site underlined.

1 mg/ml *p*-nitrophenyl phosphate in 10% diethanolamine buffer pH 9.8. The plates were incubated for 30–60 min at 37 °C, and absorbency was recorded at 405 nm using a microplate reader (Thermomax; Molecular Devices, Sunnyvale, CA, USA). Both IgG1 and IgG3 gave linear dose response curves in the assay.

ADCML assay. The ADCML studies were performed as described [10, 16, 17] Briefly, the ^{51}Cr-labelled sheep red blood cells (SRBC), target cells, were allowed to react with NIP-conjugated rabbit anti-SRBC Fab fragments with an average of 4 (NIP_4Fab) or 60 ($NIP_{60}Fab$) NIP groups per Fab fragment depending on the conjugation procedure. The total amount of NIP introduced to the surface of 1×10^8 SRBC was 80–2000 ng, respectively, depending on the amount of Fab used during the sensibilization procedure. Parallel experiments were also performed using NP (4-hydroxy-3-nitro-phenacetyl).

Variable source of complement. As human complement was used serum from a human volunteer, absorbed at 0 °C with sheep red blood cells (SRBC) to remove antibodies against SRBC. All sera used as complement source were stored in aliquots at −70 °C until use. The dilution used of human serum was 1:30 (1:90 final dilution in the test). Rabbit serum (Pel-Freez, Rogers, AR, USA) was used in 1:5 (1:15 final dilution) and guinea pig (from the animal facility at Norwegian Institute of Public Health, Oslo) was used in 1:80 (1:240 final dilution). The complement sera were split in aliquots and stored at −70 °C until use. The final suspension of target cells used in the test was about $2–3 \times 10^7$ cells/ml. Serial dilutions of isolated IgG preparations were then added to the target cell suspension. Simultaneously, the antibody concentration in the supernatants was determined by ELISA method using wells coated with $NIP_{16}BSA$ as previously described [18]. The cytotoxic index (CI) was calculated according to the formula:

$$\%CI = [(\text{cpm test} - \text{cpm spontaneous})/(\text{cpm max} - \text{cpm spontaneous})] \times 100.$$

ELISA measurements of complement activation. Complement activation was also measured in an ELISA system using microtitre plates coated with $NIP_{16}BSA$ and developed with anti-C1q [19], anti-C3 or anti-C5. Briefly, the coated ELISA plates were washed five times (SkanWasher 300) with PBS pH 7.3 with 0.05% Tween 20 (PBS/T) and incubated for 1.5 h at 37 °C with serial dilutions of cell supernatants containing NIP-antibodies (30–3000 ng/ml) in PBS/T. After washing the plates five times with PBS/T and one time with distilled water, the microtitre wells were added 100 µl 1:200 dilution of human (serum stored at −70 °C) diluted in 0.1 M Veronal buffer containing 0.25 mM $CaCl_2$ and 0.8 mM $MgCl_2$ pH 7.2 and incubated at 37 °C for 30–45 min. Then the plates were washed five times with PBS/T and added 1:4000 dilution (in PBS/T) of rabbit anti-human C5 (Dako, Copenhagen, Denmark), or 1:10000 dilution of rabbit anti-human C3c or C1q (both from Dako) and incubated for 1.5 h at 37 °C. After five times washing, the plates were added a mixture of optimum dilution of biotin labelled, locally produced sheep anti rabbit IgG, biotin-labelled alkaline phosphatase and streptavidin. The plates were incubated for 1.5 h at 37 °C, before washing five times and final incubation with the NPP substrate, and reading the absorbency at 405 nm after various times of incubation.

Results

Construction of mutant IgG1 and IgG3 antibodies

Based on available data on the complement activation site on [5, 7, 8, 20–23] we mutated the residues L234, L235, D270, K322 and P329 to alanine, P331 was mutated to glycine. All these residues are identical for IgG1 and IgG3 molecules and they are in or close to the proposed C1q binding epicentre (Fig. 1). Fig. 2 shows the cloning strategy. The cell line 293E was co-transfected with a vector containing the mutated γ1 or γ3 heavy chains and a vector containing the mouse λ1 chain. Cell supernatant was collected and quantitated for content of mutated IgG1 or IgG3 molecules. A mutant of IgG3 having a truncated hinge resembling that of IgG1 was constructed as previously described [8]. From this construct, the mutants IgG3h1K322A and IgG3h1D270A were constructed.

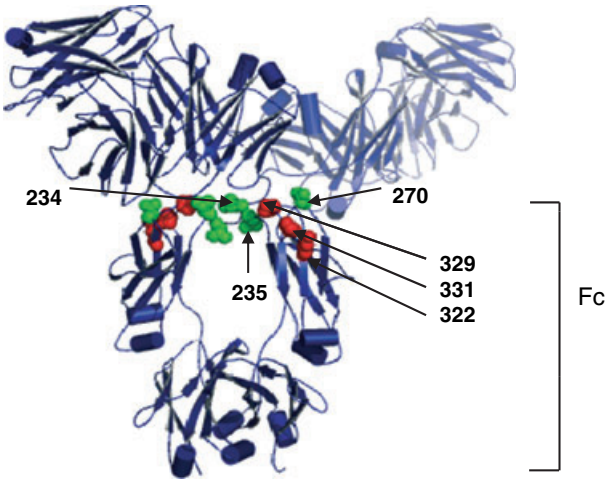

Figure 1 Crystal structure of human IgG1 b12 (PDB accession number 1HZH). The epicentre of C1q binding is highlighted in red and involves lysine 322, proline 329 and proline 331. The C1q influencing residues, as defined in this study, is highlighted in green, and involves residues leucine 234, leucine 235 and asparagine 270 (EU numbering). All residues highlighted in the figure are the same in human IgG1 and human IgG3. The overall crystallographic structure of human IgG3 is not known, but must differ at least in the hinge region which is four times larger in IgG3 compared to IgG1 [30, 31].

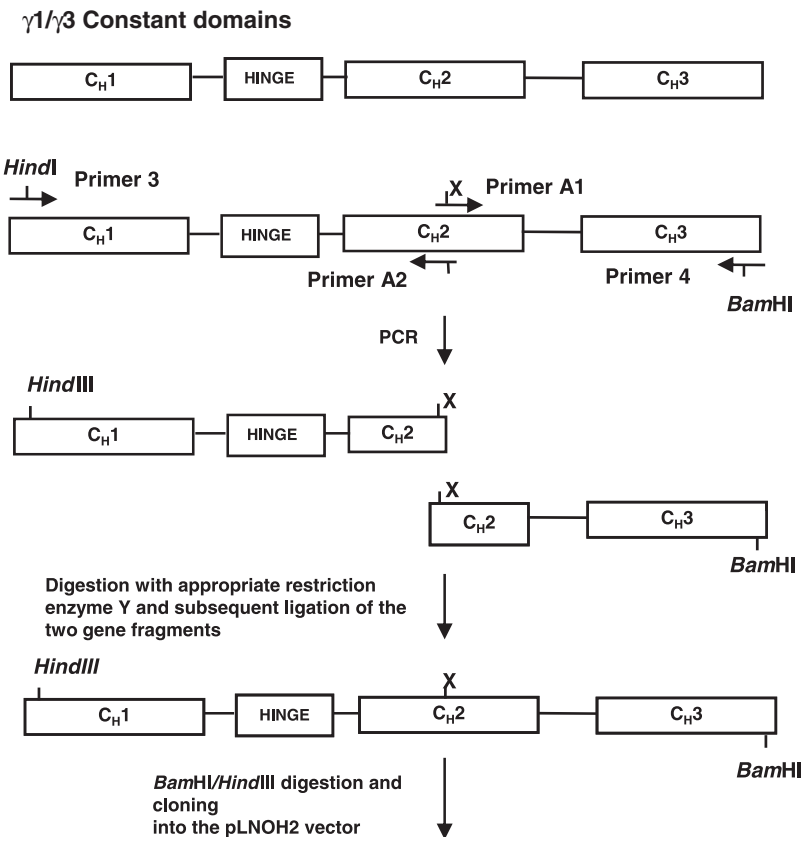

Figure 2 The gene coding for the constant part of IgG1 or IgG3 was amplified by PCR to give two overlapping gene fragments using the two primer pairs Forward IgG/mutation X A-1 (1,2) and Reverse IgG/mutation XA-2 (3,4). PCR amplifications were done using Expand high fidelity PCR polymerase (Roche, Mannerheim, Germany) applying standard conditions. The PCR products were purified on Amersham SR400 MicroSpin colums, digested with *Sfi*I or *Bse*AI, and separated by agarose gel electrophoresis. The correct DNA fragments were purified from the gel, ligated using T4 DNA ligase and introduced in the PLNOH2 vector on *Hind*III/*Bam*HI sites [12] to create pLNOH2γ1P329A. Gene sequences were verified by sequencing.

ADCML activity of mutations at residues K322, P329 and P331

The supernatants of 293E cells transfected with mutations in the γ1 and γ3 heavy chains of residues K322 and P329 to alanine and residue P331 to glycine, were quantitated for the content of IgG and used to measure the ADCML activity both at high and low antigen concentration on the target cell. Mutations at these residues abolished the ADCML activity equally for the IgG1 mutants and IgG3 mutants, except for mutant IgG3h1K322A which was positive in ADCML at high antigen concentration, while negative at low antigen concentration compared with IgG3K322A, which was negative at both situations (Fig. 3). The IgG3h1 has enhanced ADCML activity compared to wild type IgG3 as also previously published for IgG3m15 which is an mutant IgG3 with 15 aa hinge compared to 62 aa hinge of wild type IgG3 [24] Residues K322, P329 and P331 revealed no IgG subclass specific differences and muta-

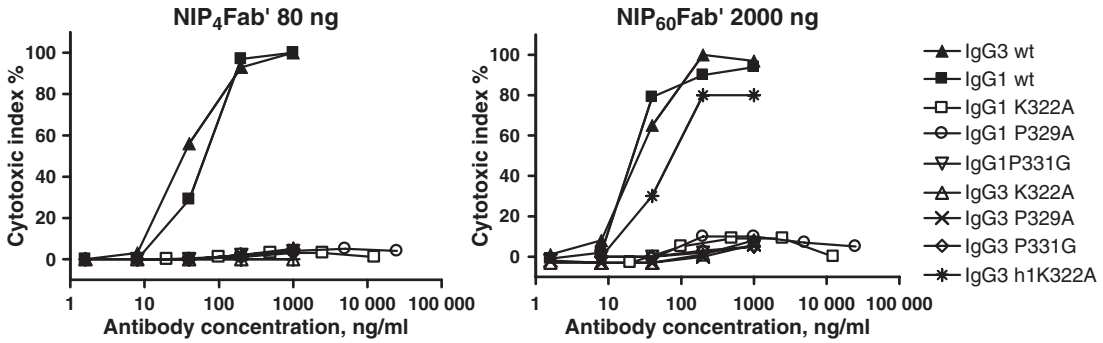

Figure 3 Antibody-dependent complement-mediated lysis activity of IgG1 and IgG3 K322A, P329A, P331G as well as IgG3h1K322A mutants tested at low (left panel) and high (right panel) NIP antigen concentration on SRBC target cells using human serum as complement source. The analysis was done in duplicates. The variation between the duplicates was less than 5% and thus would not be visible on the plots. All experiments were performed at least three times with essentially the same results. The IgG3h1K322A mutant is negative at low antigen concentration while its symbol is invisible due to overlap with other symbols.

tions of them behaved equally for otherwise wild type IgG1 and IgG3 antibodies.

ADCML activity of mutations at residues L234, L235 and D270

Supernatants of 293E cells transfected with mutations in the $\gamma 1$ and $\gamma 3$ heavy chains of residues L234, L235 and D270 to alanine were quantitated for the content of IgG and used to measure the ADCML activity both at high and low antigen concentration on the target cell.

The mutant IgG molecules were first tested for ADCML activity at low NIP antigen concentration and at low patchiness (how many NIP groups are attached to the Fab anti-sheep red blood cells) on the target cell, and the results demonstrated that the IgG1 molecules were heavily suppressed in activity compared to the wild type, while the parallel IgG3 mutants were only moderately reduced in activity compared to the IgG3 wild type molecules. This effect was demonstrated for all three residues, but the most dramatic effect was seen by the D270A mutation where the ADCML activity of the IgG1 D270A mutant was almost totally abolished (Fig. 4 upper panel).

The IgG mutant antibodies were also tested at high antigen concentration and patchiness on the target cells. The results in ADCML showed that the differences between the IgG1 mutants and the IgG3 mutants were much smaller when testing against target cells with high antigen concentration and patchiness (Fig. 4, lower panel). However, there was still a considerable difference even at high antigen concentration for the IgG1D270A mutant which was heavily reduced in ADCML compared to the IgG3D270A mutant (Fig. 4, lower panel).

Influence of the complement source upon the differences between the IgG1 and IgG3 mutants

It has been reported that the complement source used in lytic assays can have a major influence on the results in some test systems. We therefore tested the wild type IgG1 and IgG3 and their mutants in parallel using rabbit, guinea pig as well as human sera as complement source. The results showed that the serum source had a major influence on the results (Fig. 5). For the L234A and L235A mutations it seemed that human serum as complement source revealed the largest differences between the IgG1 and IgG3 mutants, guinea pig serum revealed intermediary differences and rabbit serum the least differences (Fig. 5A,B). Furthermore, when rabbit and guinea pig sera were used as complement source the difference between the IgG1 and IgG3 mutants were abolished when employing high antigen concentration on the target cells (Fig. 5A,B). Concerning the D270A mutation, guinea pig serum used as complement source revealed the largest difference between IgG1 and IgG3 showing that IgG1D270A was completely without ADCML activity at low antigen concentration and a considerable reduction even at high antigen concentration (Fig. 5C). When using rabbit serum as complement source the IgG1D270A mutant showed only a low ADCML activity at low antigen concentration (Fig. 5C lower panel). The wild type IgG1 and IgG3 antibodies induced ADCML differently depending on antigen concentration on the target cell and complement source.

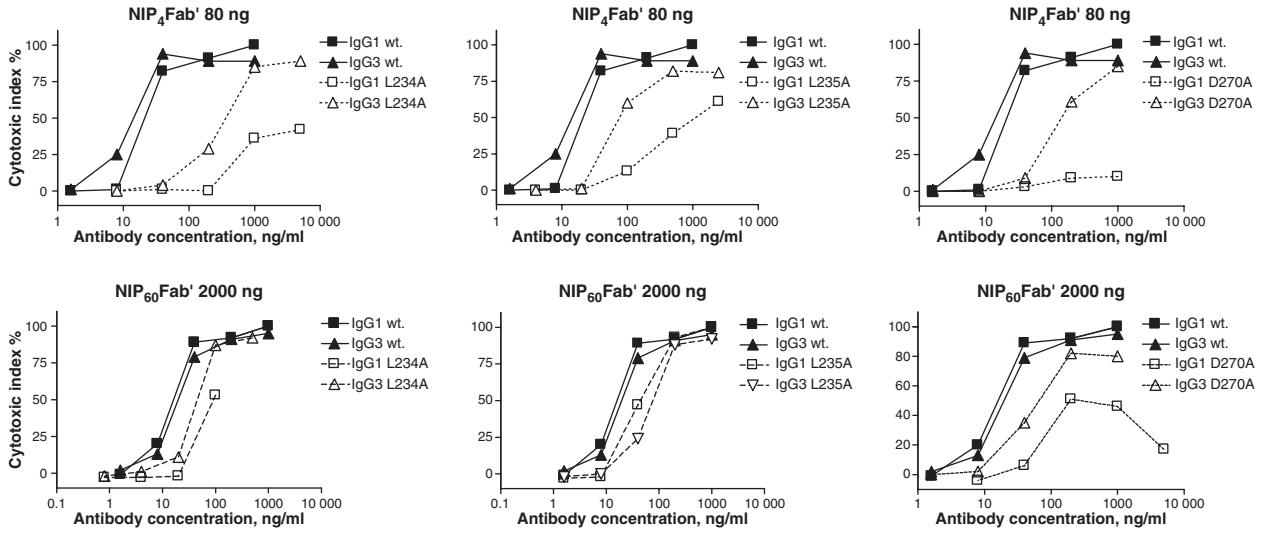

Figure 4 Antibody-dependent complement-mediated lysis activity of IgG1 and IgG3 L234A, L235A and D270A mutants tested at low NIP antigen concentration on SRBC target cells using human serum as complement source. The analysis was done in duplicates. The variation between the duplicates was less than 5% and thus would not be visible on the plots. All experiments were performed at least three times with essentially the same results.

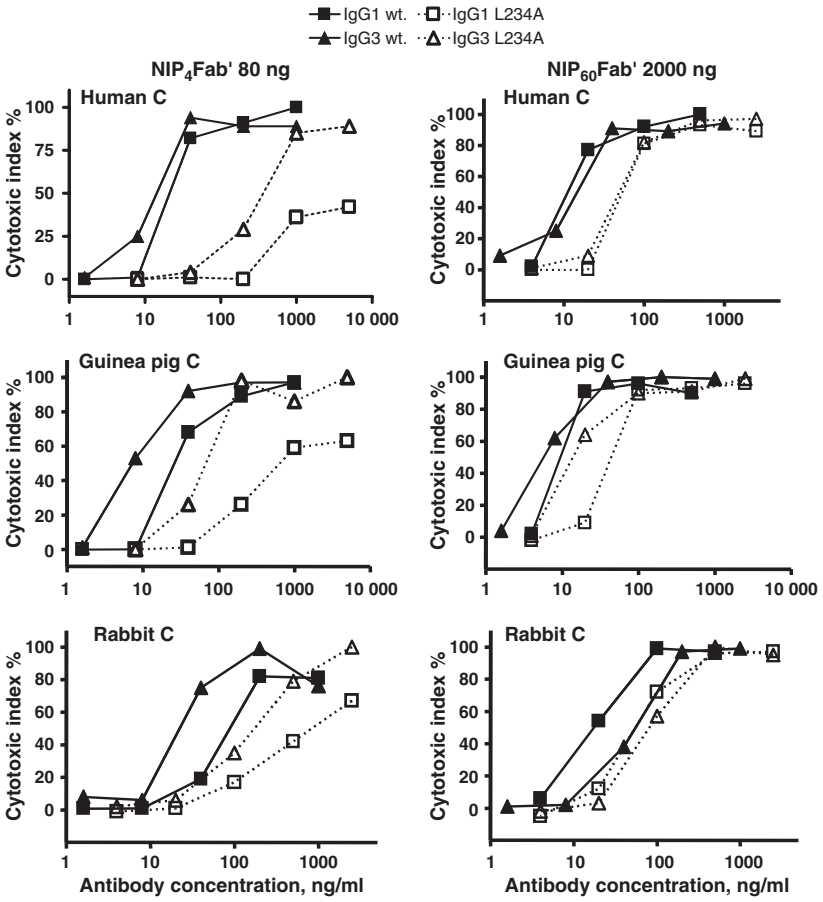

Figure 5 Antibody-dependent complement-mediated lysis activity of IgG1 and IgG3 L234A (A), L235A (B) and D270A (C) mutants tested at low and high NIP antigen concentration on SRBC target cells using human serum, guinea pig serum and rabbit serum as complement source. The analysis was done in duplicates. The variation between the duplicates was less than 5% and thus would not be visible on the plots. All experiments were performed at least three times with essentially the same results.

Thus, IgG3 was superior to IgG1 at low antigen concentration, which was reversed at high antigen concentration. This difference was relative moderate when using human serum as complement source (Fig. 5 upper panels), which we also have published previously [10], while the difference was more pronounced by using guinea pig serum and even more by using rabbit serum as complement source (Fig. 5 middle and lower panels).

Complement activation of the IgG1 and IgG3 mutants measured by ELISA

The cell supernatant of clones producing mutations at position L234, L235 and D270 in IgG1 and IgG3 was tested for complement activation by ELISA using wells coated with $NIP_{15}BSA$ and development with antibodies specific for C1q, C3c and C5 respectively. The measurement reflects complement activation at the level of C1q, C3 and C5. The results from the ELISA method showed that that the activity of the IgG1 mutants were abolished when measured at the C1q and C3 level compared to the IgG3 mutants which were reduced in activity (Fig. 6). However, when tested at the C5 level the IgG1 mutants also showed activity, although lower than the IgG3 mutants (Fig. 6, right panels). The ELISA methods did not reveal any striking difference between the D270A mutants on one hand and the L234A and L235A mutants on the other hand (Fig. 6).

Influence of antibody affinity on the ADCML activity of the mutants

The NIP-antibodies used in the study has a 10-fold higher affinity for the hapten NIP than for the cross-reacting hapten NP [25]. This enables us to test for the possible influence of antibody affinity on the observed differences between IgG1, IgG3 and their mutants. The results showed the differences between the IgG1 mutants and the IgG3 mutants were enhanced when testing against NP-sensitized target cells compared with NIP-sensitized target cells (Fig. 7). Again the differences between IgG1 and IgG3 for the L234A and L235A mutants were most pronounced when using human serum as complement source, while guinea pig serum as complement source discriminated best between IgG1 and IgG3 for the D270A mutant (Fig. 7). When probing at lower antibody affinity, the superior ADCML activity of IgG3 at low antigen concentration was most pronounced when using guinea pig serum as complement source, slightly less when using human serum and least when using rabbit serum (Fig. 7).

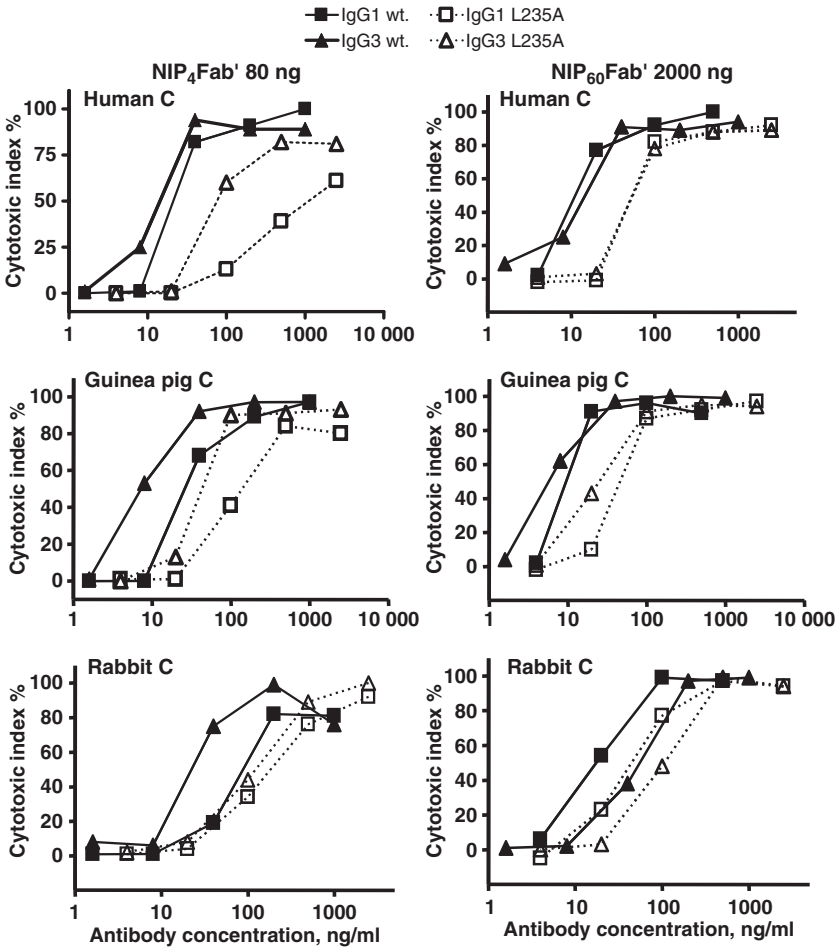

Figure 5 Continued.

Influence of the long hinge region of IgG3

The X-ray structural analysis of IgG1 indicates that the D270 residue is particularly close to the hinge and this residue might therefore influence IgG1 and IgG3 differently because IgG3 has a four times longer hinge region than IgG1. To test this possibility, we created a D270A mutant based on an IgG3 with a truncated hinge resembling that of IgG1 giving mutant IgG3h1D270A. This mutant was tested in ADCML, which revealed the same differences between IgG1 and IgG3. Thus the high ADCML activity of IgG3D270A compared to IgG1D270A was not due to the long hinge region of IgG3 compared to IgG1 as IgG3h1D270A also revealed high ADCML activity (Fig. 8).

Discussion

Hinge differences

The human IgG subclasses show an extensive structural similarity due to an amino acid sequence homology of more than 95% in the C_H2 and C_H3 domains [26]. However, the hinge region of IgG1 and IgG3 differs greatly, first recognized in the early 70 ties [27–29] and later verified as hinge length of 15 aa for IgG1 and 62 aa for IgG3 respectively [30, 31]. Intact hinge region, or at least one S–S bond at this site is necessary for complement activation [18, 32]. Apart from some integrity of the hinge is required for complement activation, the binding site for C1q is located to the hinge proximal part of the CH2 region and for murine IgG2b mapped to residues 318^{Glu}, 320^{Lys} and 322^{Lys} [20]. However, we and others have shown that for human IgG1 [7] and human IgG3 with a hinge of IgG1 [8] only lysine at residue 322 is critical for complement activation as mutations at both 318^{Glu}, 320^{Lys} do not influence complement activation. Interestingly, when the extended hinge region of IgG3 is shortened to 15 aa by hinge exon deletion, the complement activation of the mutant (IgG3m15) is dramatically increased [24]. Besides, an IgG3 double mutant with a hinge of IgG1 (IgG3h1K322A) maintained complement activation under sensitive conditions i.e. at high antigen concentration on the target cells, but lost activity at less sensitive conditions (low antigen concentration) [8]. In contrast, in the present study we show that the parallel mutant, IgG3K322A, with a normal hinge region of 62 amino

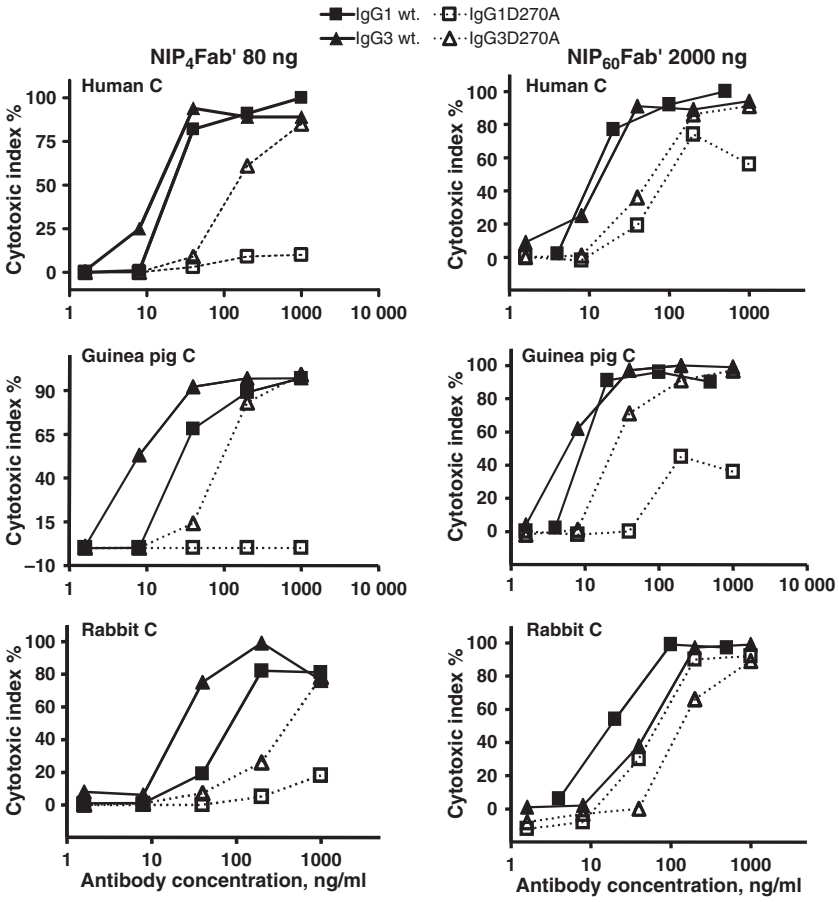

Figure 5 Continued.

acids [30, 31] is largely devoid of ADCML activity both at high and low antigen concentration on the target cell. Apparently, the extended hinge region of IgG3 down regulates the ADCML activity of the molecule by an unknown mechanism.

Residues Lys322, Pro329 and Pro331

The residues K322, P329 and P331 are crucial for complement activation for both IgG1 [5, 7, 21, 23] and verified for K322 of IgG3 [8, 23] and for P329 and P331 of IgG3 in this report. Interestingly, when mutation at position 329 was mutated from proline to alanine was performed on the basis of IgG3h1 (IgG3 with a hinge of IgG1), the resulting IgG3h1P329A molecule was devoid of ADCML activity even at high target antigen concentration [33]. Apparently residue proline 329 is even more crucial for ADCML activity, at least for IgG3, than residue lysine 322. Thus a more profound distortion of the structure of the C1q binding site was induced in the IgG3P329A mutant molecules compared to IgG3K322A, while this distortion was apparently equally influential upon ADCML for both IgG1P329A and IgG1K322A.

The mutant IgG1 and IgG3 antibodies were tested for antibody induce complement-mediated lysis by employing the versatile, flexible NIP system allowing measurements at high and low antigen concentration on the target cell. The influence of antibody affinity can also be easily tested in this system by switching between NP and NIP antigens on the target cell since these antibodies have 10-fold lower affinity for NP than for NIP [25].

Residues Asp270, Leu234 and Leu235

When residues D270, L234 and L235 were mutated to alanine, we observed a striking difference between human IgG1 and IgG3. Thus the IgG1 mutants were dramatically reduced in ADCML activity compared to the corresponding IgG3 mutants.

The difference between IgG1 and IgG3 was most prominent at low antigen concentration on the target cells, and decreased when the antigen concentration on the target cell was increased. Residue asparagine 270 was particularly discriminating, and the IgG1D270A mutant was virtually negative in ADCML, while IgG3 D270A was only partially reduced in activity at low antigen concentration. The D270 residue has been assigned to

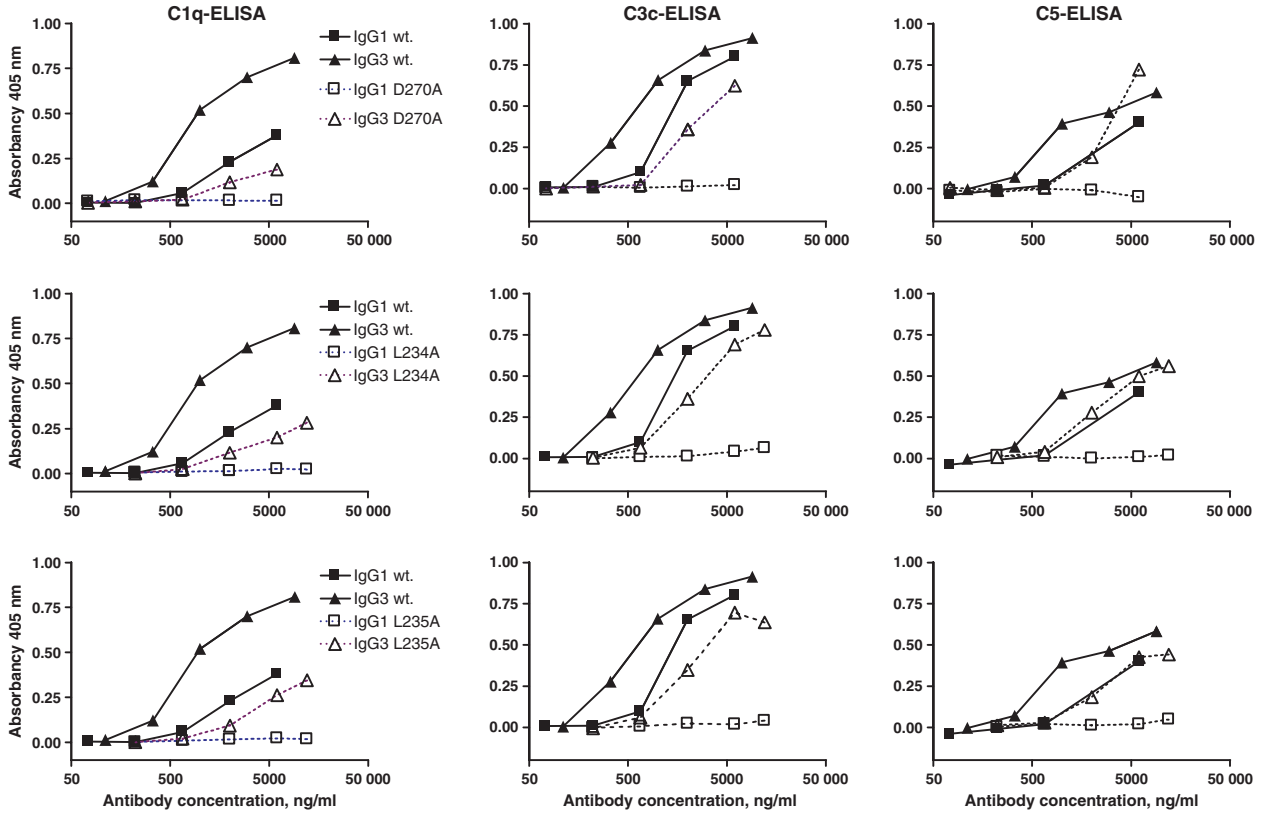

Figure 6 Complement activation of the IgG1 and IgG3 L234A, L235A and D270A mutants measured by ELISA technique using microtitre wells coated with $NIP_{16}BSA$ and developed on the level of C1q, C3 and C5, respectively. Human serum was used as complement source. Within one row, the antibodies were tested simultaneously, in duplicates and on the same ELISA-plate. The variation between the duplicates was less than 5% and thus would not be visible on the plots. The experiments were repeated three times with essentially the same results.

the epicentre of C1q binding site of IgG1 [7]. However, in the present study we showed that the IgG3 molecule is not as dependent as IgG1 on D270, thus the IgG3D270A mutant was only slightly reduced in ADCML activity at low antigen concentration. The IgG1D270A mutant described previously [7] was made on an anti-CD20 background, while we have been using the versatile anti-NIP antibody platform. This flexible platform can mimic different antigen distribution on target cells, both regarding antigen concentration and antigen patchiness as well as antibody affinity [17]. Thus, we have previously shown that IgG3wt is better than IgG1wt in ADCML activity when the antigen concentration and/or patchiness is low [10, 34] and we and others have shown that human IgG2wt efficiently activates complement when the antigen concentration on target cell is high, while they are negative at low antigen concentration [10, 11, 34].

Antigen concentration and patchiness

Both human IgG1 and IgG3 activate complement very efficiently and by using the NIP-specific antibody matched set, IgG3 performs better than IgG1 at low antigen concentration, while the differences disappear and can get even reversed at high antigen concentration [10]. The access to, distribution and concentration of antigen on the target cells will thus directly influence how the different human IgG isotypes will perform in complement activation. Apparently, the performance of the human IgG subclasses with respect to complement activation and other effector functions as well, is dependent on the conditions whereupon they act. The NIP antibody platform is ideal for scouting the wild type IgG subclass as well as mutant antibodies for alteration in effector functions. This can be translated to the specific test systems like anti-CD20 as well as other antibody specificities, depending on the concentration and distribution of the cognate antigen epitope on the target cell.

Biological relevance of IgG1 and IgG3

IgG1 and IgG3 show different kinetic during an immune response against a protein vaccine. Thus, IgG1 is often formed after the first vaccine dose, while IgG3 generally needs more than one dose, in addition, IgG3 declines more rapidly [35]. The rapid decline of human IgG3 formed after vaccination is at least partly due to the short

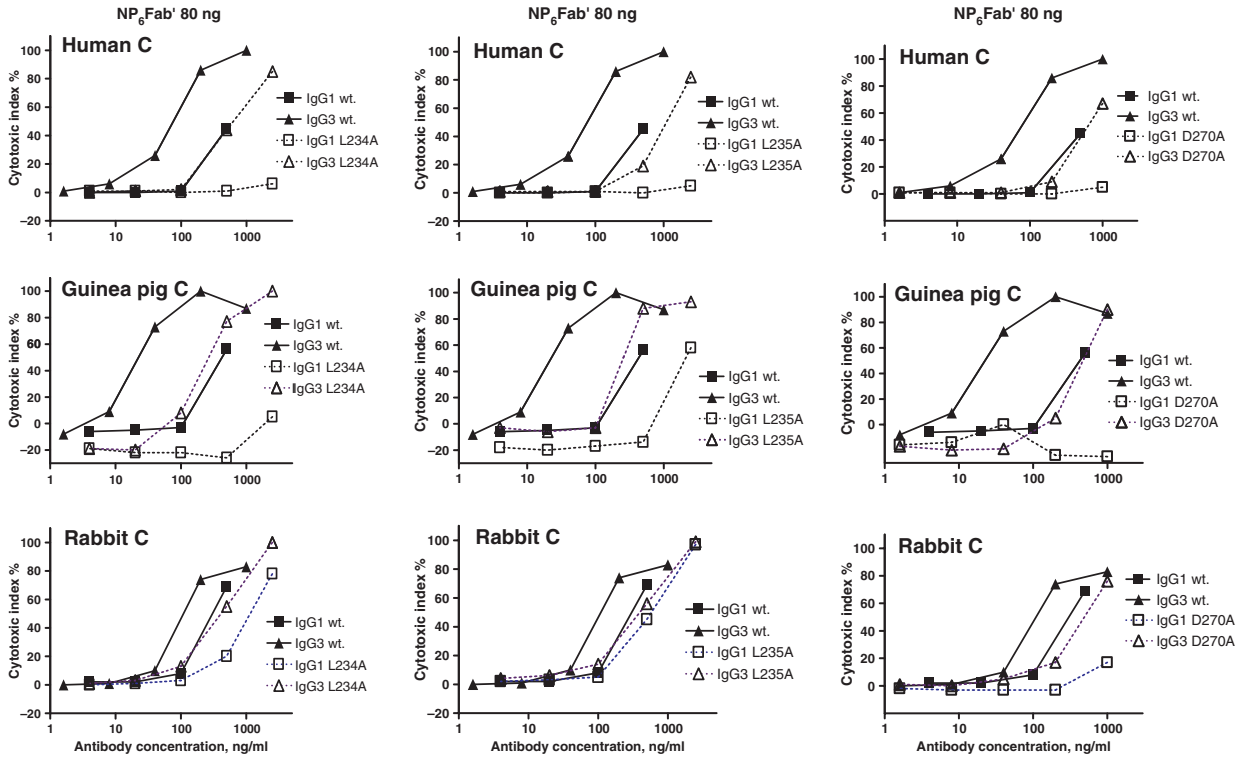

Figure 7 Antibody-dependent complement-mediated lysis activity of IgG1 and IgG3 L234A, L235A and D270A mutants tested at low NP antigen concentration on SRBC target cells using human serum, guinea pig serum or rabbit serum as complement source. The analysis was done in duplicates. The variation between the duplicates was less than 5% and thus would not be visible on the plots.

half life human IgG3 [36]. Complement activation is very important for protection against microbial pathogens [37]. However, complement activation is a powerful pro-inflammatory condition which needs strict control in order to avoid pathology. Particularly at low antigen concentration, IgG3 is superior to IgG1 in complement activation [10]. Furthermore, IgG3 has as extremely long and flexible hinge region coded by four hinge exons [30, 31, 38]. Strikingly, the complement activation capacity of IgG3 can be enhanced by hinge exon deletions [24]. The supple structural differences between human IgG1 and IgG3 were revealed by parallel site-directed mutation documented in this report adds to the previously acknowledged differences between IgG1 and IgG3. This further strengthens the notion of biological specialization of the human IgG1 and IgG3 subclasses. Accordingly, the use of either IgG1 or IgG3, wild type or mutants should be recognized when choosing IgG isotype for immune therapy, although IgG1 has up to now been the first choice [39]. Manipulating IgG3 toward IgG3R435H [as found in the G3m(st) allotype], frequent in Asians but seldom in Caucasians [40] would be particularly interesting as a longer half life of IgG3 is then expected [41].

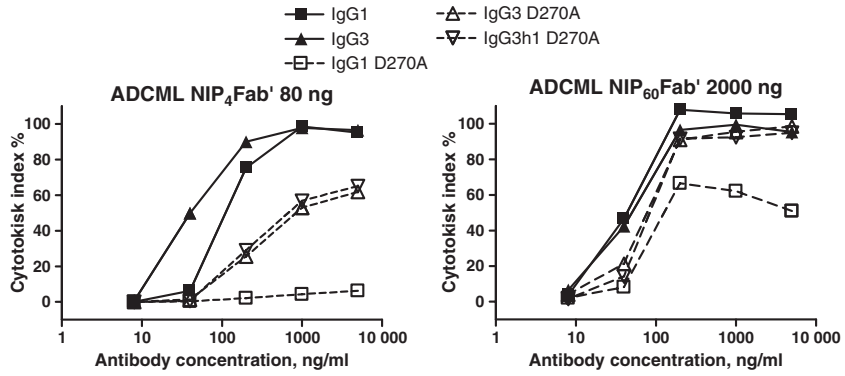

Figure 8 Antibody-dependent complement-mediated lysis activity of IgG1, IgG3 and IgG3h1D270A mutants tested at low and high NIP antigen concentration on SRBC target cells using human serum as complement source. The analysis was done in duplicates. The variation between the duplicates was less than 5% and thus would not be visible on the plots.

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