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A mutant human IgG molecule with only one C1q binding site can activate complement and induce lysis of target cells

Terje E. Michaelsen[1,2], John E. Thommesen[3], Oistein Ihle[1], Tone F. Gregers[3], Randi H. Sandin[1], Ole Henrik Brekke**[3] and Inger Sandlie[3]*

[1] Department of Vaccination and Immunity, Division of Infectious Disease Control, Norwegian Institute of Public Health, Oslo, Norway
[2] Institute of Pharmacy, University of Oslo, Oslo, Norway
[3] Department of Molecular Biosciences, University of Oslo, Oslo, Norway

There are potentially two binding sites for C1q on IgG, one on each C_H2 domain of the gamma heavy chains, close to the lower hinge region. It is not clear whether the presence and involvement of both the C1q binding sites is necessary to induce the activation signal of human IgG. In order to clarify this issue, we made a hybrid mutant IgG1/IgG3 molecule where the IgG1 half of the molecule was made unable to activate complement through the introduction of a P329A mutation. The IgG3 half of the molecule was mutated to harbor a hinge region identical to that of IgG1, and for detection a peptide tag derived from p21ras was introduced into the FG loop of the C_H1 domain. The hybrid IgG1P329A/IgG3h1-ras molecules were isolated by Protein A affinity chromatography and shown to activate complement and induce complement-mediated lysis at the same levels as wild-type IgG1 and IgG3h1-ras molecules. Thus, one C1q binding site per IgG is sufficient to induce activation. Wild-type human IgG molecules might also normally expose only one C1q binding site as already shown for interaction with FcγR, were IgG expose one binding site per molecule.

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Introduction

Antibody-dependent complement-mediated lysis (ADCML) is initiated by the binding of two or more IgG to the surface of target cells. Provided the antigen concentration and orientation is optimum, this is followed by a multivalent interaction between the IgG and C1q [1], which is part of the first component (C1) of the complement cascade. 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 C_H2 domain of human IgG demonstrate the importance, direct or indirect, of several residues, such as K322 [7, 8], P329 and others [7]. The binding sites for the FcγR (FcγRI, FcγRII and FcγRIII) are also located in the C_H2 domain and close to the lower hinge region as well [9–12]. Interestingly, the crystal structure of an FcγRIII-IgGFc complex shows that the Fc fragment is asymmetric in the complex, such that only one FcγR binding site is exposed [13].

Correspondence: Prof. Terje E. Michaelsen, Division of Infectious Disease Control, Norwegian Institute of Public Health, PO Box 4404 Nydalen, N-0403 Oslo, Norway
Fax: +47-22353605
e-mail: terje.e.michaelsen@fhi.no

Abbreviations: **ADCML:** antibody-dependent complement-mediated lysis · **ALP:** alkaline phosphatase · **gC1q:** C-terminal heterotrimeric globular IgG/IgM binding domain of C1q · **NIP:** 5-iodo-4-hydroxy-3-nitro-phenacetyl · **NPP:** 4-nitrophenyl-phosphate · **PBS/T:** PBS containing 0.05% Tween 20 · **SRBC:** sheep red blood cells

* **Present address:** Department of Biochemistry, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo, Norway
** **Present address:** Invitrogen Dynal Bead Based separations, Oslo, Norway

The C1q molecule is built from six units, each terminating in a C-terminal heterotrimeric globular IgG/IgM binding domain (gC1q), reviewed in [14, 15]. The crystal structure of the gC1q has been reported [16] and form the basis of a hypothetical model of one gC1q domain in complex with IgG [16, 17]. In this model, the amino acids already implicated in binding of gC1q to the Fc region, including K322 and P329 [7], are at or near the binding interface of the globular B domain of gC1q. C1 consists of two copies of two proteases, C1r and C1s, in addition to the recognition molecule C1q [1, 7, 17]. Binding of C1q to immune complexes is thought to elicit a mechanical stress to the C1 complex that triggers self-activation of C1r. While it is clear that multivalent binding of several gC1q "heads" to clusters of IgG molecules generates the activation signal [6, 18–20], it is uncertain whether each human IgG molecule must harbor two C1q binding sites for activation to take place. We addressed this question by constructing a hybrid human IgG molecule where the two heavy chains differed in their ability to bind C1q. One heavy chain was of IgG1 origin and had the P329A mutation that ablates C1q binding. The other chain was of IgG3 origin, while the hinge region was modified such as to mimic that of IgG1. The two heavy chains paired to form an S-S-bonded heteromonomer, and we were able to purify the hybrid IgG by affinity chromatography on a Protein A column. The hybrid molecule was able to activate complement, and thus the involvement of only one C1q binding site per human IgG molecule is sufficient for complement activation. It is also conceivable that wild-type IgG also normally expose only one C1q binding site and that this is sufficient for complement activation.

Results

Construction of hybrid IgG molecules

Figure 1 shows the cloning strategy. The IgG1P329A mutant was compared with IgG1 wild type in an ADCML assay. As expected and as reported by others [7], the

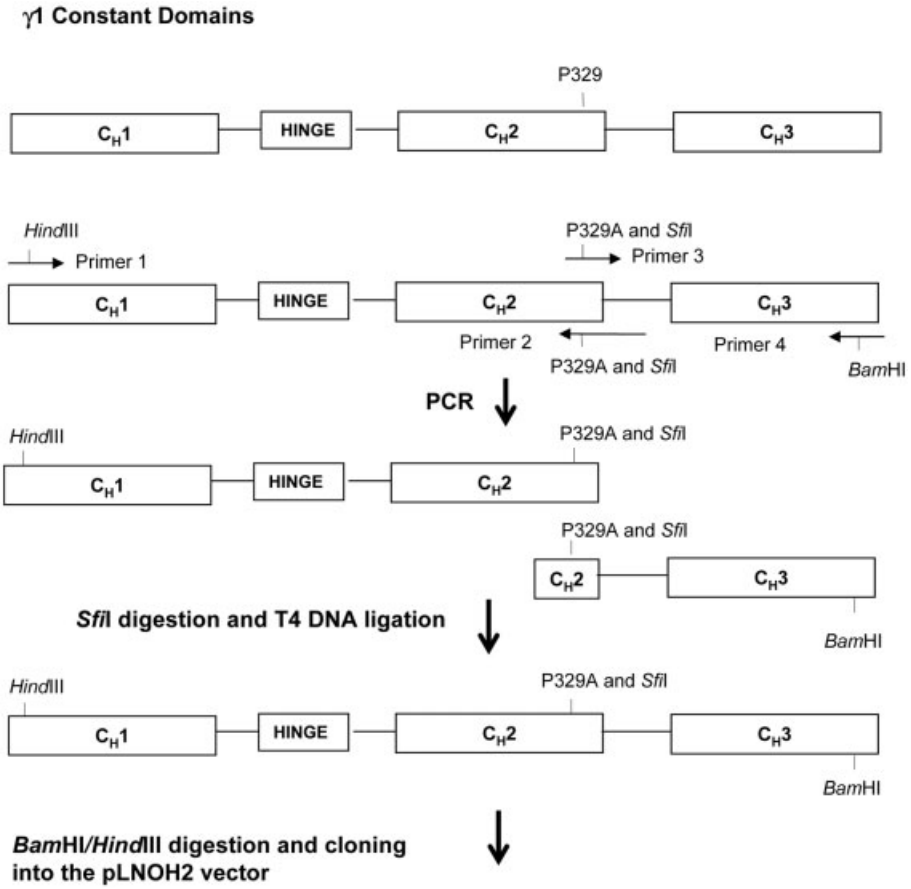

Figure 1. The gene encoding the constant part of IgG1 was amplified by PCR to give two overlapping gene fragments using two primer pairs: primers 1 + 2 and primers 3 + 4. The two resulting PCR products had *Sfi*I restriction sites introduced by silent mutation and the P329A mutation. The γ1P329A constant region gene was constructed by *Sfi*I digestion and subsequent joining of the two resulting gene fragments. Flanking *Bam*HI and *Hind*III restriction sites were introduced by primers 1 and 4.

P329A mutation impaired the ability of IgG1 to induce ADCML. Here we show this impairment at both high and low antigen densities on target cells (Fig. 2). To create a hybrid, we transfected J558L cells with a vector containing this mutant γ1 heavy chain gene as well as a gene encoding a mutant γ3 heavy chain. The hinge region of the γ3 heavy chain was mutated to that of the γ1 heavy chain, and the location of the disulfide bonds between the heavy and light chains found in IgG1 was made possible by introduction of a C131S mutation in the $\gamma 3C_H1$ domain [21]. Furthermore, a tag peptide sequence (KLVVVGAGGVGKSALTI) derived from p21ras with a G12V mutation [22] was introduced into the FG loop of the C_H1 domain, and we verified that the mutation did not alter the complement activation nor the ADCML activity of the corresponding IgG3h1-ras molecule (documented later).

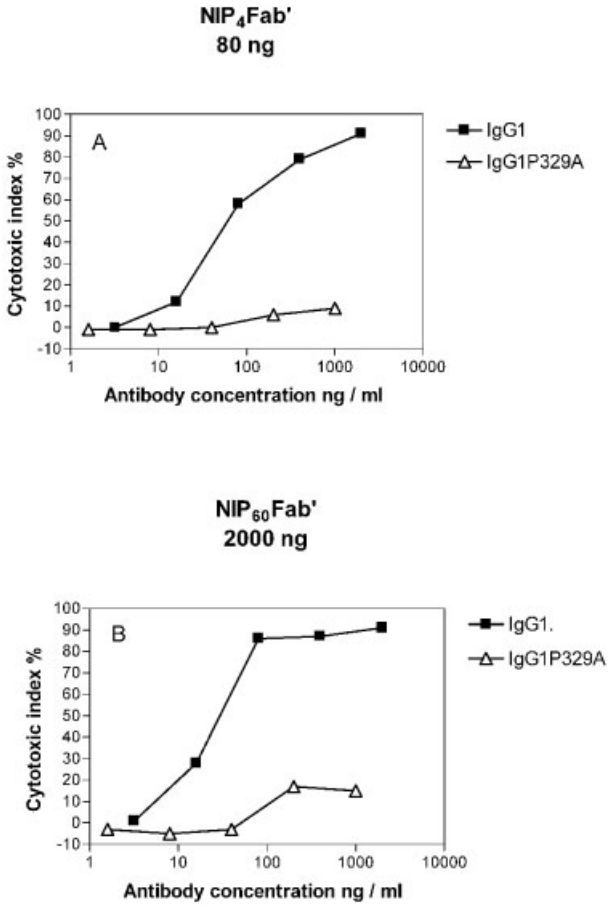

Figure 2. ADCML activity of IgG1 wild type and IgG1P329A. The experiments were done in duplicate at low (A) and high (B) antigen concentration and patchiness on target SRBC with human serum as the complement source. The variation between duplicates was less than 5% and thus is not visible on the plots. All experiments were performed at least three times with essentially the same result.

Screening transfectants for the production of hybrid IgG1P329A/IgG3h1-ras molecules

As Protein A interacts with the IgG1 half of the hybrid and the IgG3 half has a ras-tag, an ELISA detection system was designed as follows: Protein A was coated onto plates to catch the IgG1P329A half of the hybrid. After incubation with various test samples followed by a washing step, a ras-specific mouse IgG2b mAb was added to the plates. Since IgG3h1-ras does not bind Protein A [23] and is removed in the previous washing step, this anti-ras mAb can only react with the hybrid IgG1P329A/IgG3h1-ras molecules. Thus, this ELISA test system could be used to screen for the presence of clones that produced hybrids. The test system was applied to transfectants subjected to limiting dilution that had more than 5 μg/mL total IgG in the cell supernatant.

Isolation of IgG1P329A/IgG3h1-ras hybrid molecules by Protein A affinity chromatography

We established a purification system based on Protein A chromatography that separated the three-component mixture of 1) homomonomeric IgG3h1-ras, 2) homomonomeric IgG1P329A and 3) heteromonomeric hybrid IgG1P329A/IgG3h1-ras molecules. Since Protein A binds human IgG1 but not IgG3 [23], we anticipated that the hybrid IgG1P329A/IgG3h1-ras would have an intermediate affinity to Protein A and could therefore be eluted from a Protein A column at a pH between 7.0 and 4.0. A similar hybrid between mouse IgG1 and mouse IgG2a could be eluted at pH 5.4, while the parental IgG1 and IgG2a eluted at pH 6.0 and 4.9, respectively [24]. The cell supernatants from the selected clones were therefore subjected to affinity chromatography on a Protein A column, as were control cell clones producing homomonomers of IgG1P329A or IgG3h1. The most efficient way to obtain pure hybrid molecules in high yield turned out to be a stepwise approach using five buffers with pH values of 7.3, 6.0, 4.7, 4.5 or 4.0. Fractions were collected and processed as described in the Materials and methods, and the chromatogram obtained is shown in Fig. 3.

In step 1, fraction 1 was eluted at pH 7.3. Naturally, this fraction contained IgG3 h1-ras that did not bind Protein A, while step 2 at pH 6.0 removed loosely bound IgG3 h1-ras. In step 3, a peak was eluted at pH 4.7 that did not appear when cell clones producing homomonomers of IgG3h1 or IgG1P329A were applied to the column. Most importantly, less than 0.1% of IgG3h1 and only about 1–5% of IgG1P329A molecules were eluted at pH 4.7 (data not shown). Fraction 4 was eluted at pH 4.5, while fraction 5 was eluted at pH 4.0.

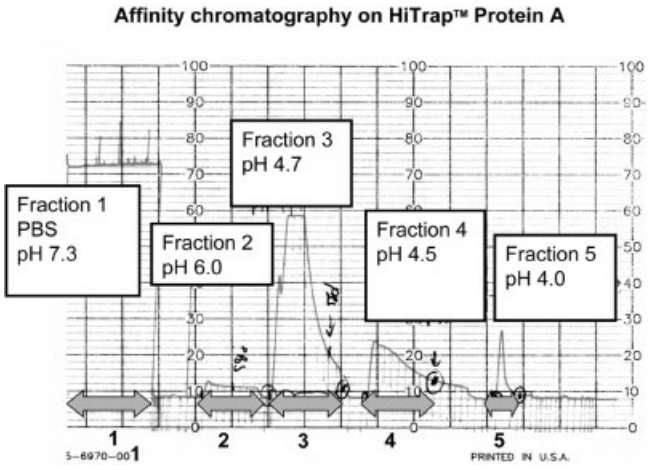

Figure 3. Affinity chromatography of supernatant from a cell clone producing a mixture of IgG3h1-ras, IgG1P329A/IgG3 h1-ras hybrid and IgG1P329A molecules. Portions of 1900 mL cell supernatant were applied to a 5 mL HiTrap Protein A column and eluted at a rate of 1–1.5 mL/min. Unbound material containing IgG3h1-ras and culture medium was eluted with PBS at pH 7.3 (fraction 1). The column was subsequently eluted with 0.2 M citrate buffer at pH 6.0 (fraction 2), 0.2 M acetate buffer at pH 4.7 (fraction 3), 0.2 M acetate buffer at pH 4.5 (fraction 4) and 0.2 M acetate buffer at pH 4.0 (fraction 5). The column was finally washed with 0.2 M acetic acid followed by reconstitution with PBS at pH 7.3. The figure is based on scanning of the original chromatogram.

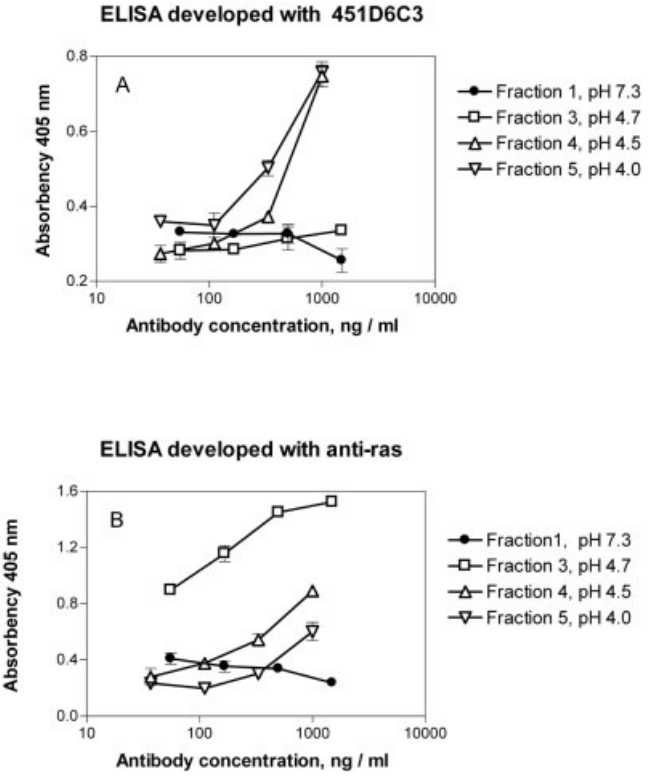

Figure 4. ELISA of fractions eluted from the Protein A column using microtiter plates coated with Protein A (2 μg/mL when developed with anti-ras and 0.2 μg/mL when developed with mAb 451D6C3). Increasing concentrations of antibodies from fractions 1, 3, 4 and 5 were added and detected with either mAb 451D6C3, which reacts with IgG1 (A), or anti-ras mAb, which reacts with IgG3h1-ras (B). The tests were done in duplicate and the variations are shown.

Immunological characterization of the eluted fractions

The concentration of IgG in each fraction, irrespective of subclass, was measured by testing serial dilutions in ELISA wells coated with antigen ($NIP_{15}BSA$); both heavy chain genes had exons encoding a murine variable domain with specificity for the hapten 5-iodo-4-hydroxy-3-nitro-phenacetyl (NIP). The ELISA was developed with sheep anti-human IgG as described in the Materials and methods. All fractions were further analyzed by employing ELISA plates coated with Protein A. These were developed with either mAb 451D6C3 (detects IgG1P329A) or the anti-ras mAb, and the dose response curves were measured (Fig. 4).

Both homomonomeric IgG1P329A and the γ1 heavy chain of the hybrid IgG can bind to Protein A-coated wells. However, the trapped IgG1P329A molecule is detected with the mAb 451D6C3, while the hybrid IgG1P329A/IgG3h1-ras is detected with anti-ras mAb. Fraction 1 was found to contain only IgG3h1-ras antibodies. These molecules bound to ELISA wells coated with $NIP_{15}BSA$ and gave a positive signal when developed with sheep anti-human IgG and a negative signal when developed with mAb 451D6C3 (data not shown). However, the IgG in fraction 1 was not able to bind to the Protein A column and was consequently negative in all tests involving Protein A-coated plates (Fig. 4).

Antibodies eluted at pH 4.7 (fraction 3, Fig. 3) bound ELISA plates coated with Protein A and were recognized by anti-ras mAb (Fig. 4B) but not by mAb 451D6C3 (Fig. 4A). This finding indicates that fraction 3 contained hybrid molecules; the hybrid molecules do not bind mAb 451D6C3 in this experiment because the hybrid has only one binding site for mAb 451D6C3, and this site is occupied by the Protein A used to bind the hybrid molecule to the ELISA plate. The binding sites on IgG1 for mAb 451D6C3 and Protein A are highly overlapping, and they compete efficiently with each other when using IgG1 as target (T. E. M., unpublished data). Most importantly, homomonomeric IgG3h1-ras was not found in fraction 3. Thus, we conclude that fraction 3 contained highly pure hybrid IgG1P329A/IgG3h1-ras molecules. Possible contamination with the other species would be less than 0.1% for IgG3h1-ras and 1–5% for IgG1P329A based on Protein A chromatography of IgG3h1 control molecules and the results of the ELISA measurements shown in Fig. 4A. This

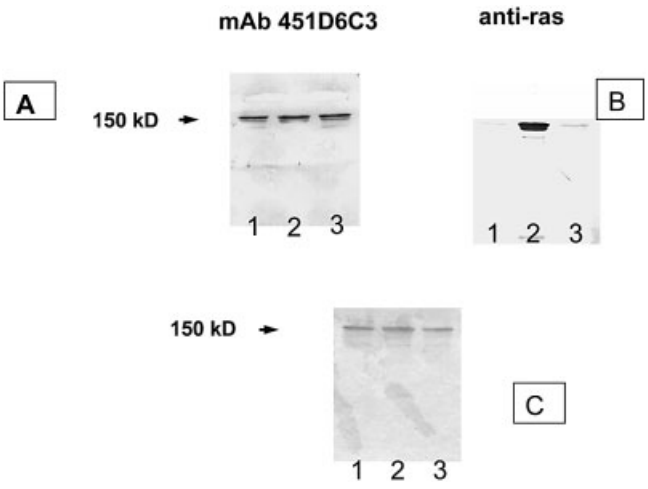

Figure 5. SDS-PAGE and immunoblot analysis of fraction 5 (IgG1P329A; lane 1), fraction 3 (IgG1P329A/IgG3h1-ras; lane 2) and purified control IgG1P329A (lane 3). Panels (A) and (B) show an immunoblot from SDS-PAGE developed with mAb 451D6C3 and anti-ras, respectively. Panel (C) shows Coomassie blue-stained SDS-PAGE. The position of molecules corresponding to a MW of 150 kDa is shown by arrows.

conclusion was further supported by the results of an immunoblot where the antibody molecules in fraction 3 were recognized by both mAb 451D6C3 and the anti-ras mAb (Fig. 5). Furthermore, Fig. 5 demonstrates that the hybrid IgG1P329A/IgG3h1-ras molecules have a molecular weight corresponding to a monomeric IgG molecule consisting of two heavy and two light chains disulfide bonded to each other.

The antibodies eluted in fraction 4 (pH 4.5) were strongly positive in the Protein A ELISA when developed with mAb 451D6C3 (Fig. 4A) and also slightly positive when developed with anti-ras (Fig. 4B). Thus, this fraction contained a mixture of IgG1P329A/IgG3h1-ras (minor part) and IgG1P329A homomonomer (major part). Finally, fraction 5 (pH 4.0) contained almost pure IgG1P329A, since it reacted strongly in the Protein A ELISA developed with mAb 451D6C3 (Fig. 4A) and reacted only faintly with anti-ras mAb (Fig. 4B). This conclusion was verified by immunoblotting, which showed the presence of intact disulfide-bonded hybrid IgG molecules with a molecular weight of 150 kD in fraction 3, and these molecules reacted with both anti-ras and mAb 451C6D3 (Fig. 5). An estimate of the relative distribution of the three antibody molecules in the cell culture media based on the elution profiles from the Protein A column was approximately 45–50% for IgG3h1-ras, 35–45% for IgG1P329A/IgG3h1-ras hybrids and 10–15% for IgG1P329A.

The IgG1P329A/IgG3h1-ras hybrids activate the complement cascade

The antibodies eluted in fractions 1, 3, 4 and 5 were tested in ELISA assays for complement activation. Dilutions of antibodies were incubated together with human serum as a complement source, and the plates were developed with antibodies specific for C1q, C3c or C5. Thus, the measurements should reflect C1q binding as well as complement activation down to the levels of C3 and C5. The results show that the hybrid IgG1P329A/IgG3h1-ras eluted in fraction 3 activated complement at approximately the same level as IgG3h1 control protein as well as IgG3h1-ras eluted in fraction 1 (Fig. 6). However, molecules eluted in fractions 4 and 5 did not activate complement, nor did IgG1P329A, as expected (Fig. 6). Thus, we demonstrated that human IgG needs only one C1q binding site per molecule to mediate this effector function.

The IgG1P329A/IgG3h1-ras hybrids induce complement-mediated lysis of target cells

The fractions eluted from the Protein A column were also tested for their ability to induce complement-mediated lysis of sheep red blood cells. As expected, fraction 1, containing IgG3h1-ras, was fully active in ADCML at both low and high antigen concentrations on the target cells (Fig. 7), at apparently the same level as IgG3h1. Thus, as anticipated, introduction of the ras-tag did not influence the complement activation activity. Fraction 3, containing highly pure IgG1P329A/IgG3h1-ras hybrid molecules, was fully active in ADCML, at a level similar to IgG3h1-ras (Fig. 7). Therefore, only one of the heavy chains in the hybrid IgG1P329A/IgG3h1-ras molecule needs a C1q binding site for IgG to be active in ADCML. Fraction 4, containing IgG1P329A contaminated with 1–5% IgG1P329A/IgG3h1-ras hybrid molecules, was negative in ADCML at a low antigen concentration (Fig. 7A) and only slightly active at a high antigen concentration (Fig. 7B), while fraction 5, containing IgG1P329A was inactive at both antigen concentrations, as expected.

Discussion

The aim of this report was to investigate whether intact human IgG molecules need two C1q binding sites to activate complement, and we show that the presence of only one C1q binding site on the IgG molecule is sufficient. It is conceivable that wild-type human IgG also involve or expose only one C1q binding site for activation of the classical complement pathway, as they do for binding to FcγR.

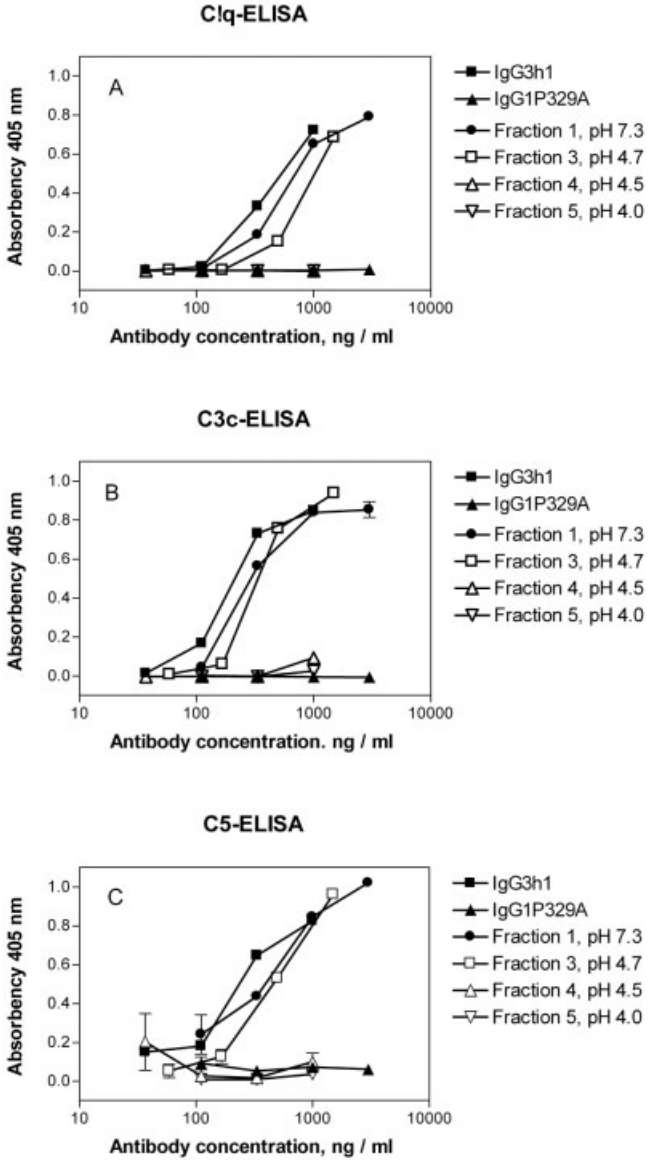

Figure 6. C1q binding and complement activation induced by IgG fractionated on Protein A column as detected by ELISA. Microtiter plates were coated with 1 μg/mL $NIP_{15}BSA$ and incubated with serial dilutions of antibody. The wells were further developed with rabbit anti-C1q (A), rabbit anti-C3c (B) or rabbit anti-C5 (C). Fraction 1 contains IgG3h1-ras, fraction 3 contains the hybrid molecules IgG1P320A/IgG3h1-ras, fraction 4 contains IgG1P329A slightly contaminated with the hybrid molecules and fraction 5 contains IgG1P329A.

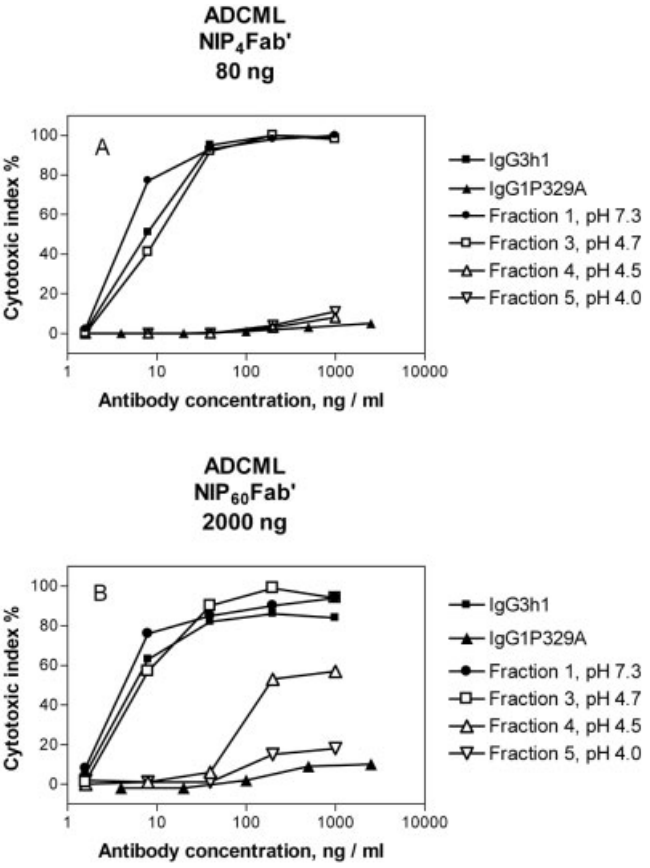

Figure 7. ADCML induced by IgG fractionated on a Protein A column. The experiments were done at low (A) and high (B) antigen concentrations and patchiness on the target cells, SRBC, using human serum as the complement source. The analyses were done in duplicate. The variation between the duplicates was less than 5% and thus is not visible on the plots. All experiments were performed at least three times with essentially the same results. Fraction 1 contains IgG3h1-ras, fraction 3 contains the hybrid IgG1P320A/IgG3h1-ras, fraction 4 contains IgG1P329A slightly contaminated with the hybrid molecules and fraction 5 contains IgG1P329A.

The interaction site for C1q on IgG is located to the upper part of the C_H2 domain, near the hinge region where the two heavy chains are covalently linked by disulfide bonds [7, 8, 25, 26], and these disulfide bonds must be intact [27–30]. Epitope density on the target cell surface, testable in our system [31], is also important for complement activation, underscored by the fact that human IgG2 is unable to activate complement at low epitope density, while it does so at high epitope density [32, 33].

We confirm in this report that NIP-specific human IgG1 antibodies depend on the P329 residue in C_H2 for complement activation, as previously reported for CD20 antibodies [7], and this is the case at both high and low antigen concentrations on the target cells. In contrast, the IgG3h1 K322A tested earlier is negative at low but positive at high antigen concentrations [8]. Consequently, we decided to mutate the human γ1 to γ1P329A in order to construct a hybrid molecule with only one C1q binding site. This hybrid IgG molecule was made up by the γ1P239A heavy chain paired with a γ3h1-ras heavy chain. We stably transfected light chain-producing plasma cells with a vector harboring the two heavy chain genes, and transfectants secreting hybrid molecules in addition to the two homomonomers were selected and expanded. The hybrid IgG molecules (IgG1P329A/IgG3h1-ras) exhibited, as expected, inter-

mediate affinity for Protein A compared to the two homomonomers, enabling us to isolate hybrid molecules by Protein A affinity chromatography by elution at pH 4.7. Since the heavy chains in the hybrid had identical hinge regions (that of IgG1) and bound light chains in the same manner, the hybrid molecules consisted of S-S-linked heteromonomeric molecules (Fig. 5). It was essential that the hybrids were free from contamination with ADCML-competent homomonomeric IgG3h1-ras molecules. Since the IgG3h1-ras molecules were eluted from the Protein A column at pH 7.3 and any residual molecules were removed at pH 6.0, contamination could be avoided. Thus, the ADCML activity of hybrid IgG1P329A/IgG3h1-ras molecules was not due to contamination with homomonomeric IgG3h1-ras molecules.

We used an artificial hybrid molecule composed of IgG3 and IgG1 heavy chains. The two molecules are closely similar in structure in the C_H2 and C_H3 domains, so it is reasonable to expect the same intermolecular interactions in the hybrid molecules as in the wild-type molecules. The greatest difference between IgG1 and IgG3 is located in the hinge region, and the IgG3 in the hybrid molecule was mutated to resemble IgG1. The hybrid molecule is thus expected to be very similar to the IgG3h1-ras molecule in structure. It is also reasonable to expect that the P329A mutation had the same negative influence on the affected heavy chains of both IgG1 and IgG1/IgG3 hybrid molecules.

A hybrid mouse IgG1/IgG2a derived by hybridoma technology has previously been tested for complement activation [34] and was found to be 50% less effective in C1q binding than the parental IgG2a; IgG1 is more or less inactive in complement activation [34]. However, it was not clear how the heavy chains were linked and whether correct disulfide bonds were present; due to the different hinge regions of mouse IgG1 and IgG2a (only 30% homology), it might be anticipated that the intermolecular interactions were not correct [35].

It is the globular heads of C1q (gC1q) that bind to IgG and initiate the activation of complement. The model of the gC1q:IgG interaction based on the crystal structure of gC1q [16] as well as the crystal structure of IgG1 b12 [36] features gC1q binding at the Fab-Fc interface [14], thus in addition to binding to C_H2, gC1q is in contact with Fab. This underscores a critical role for the hinge region in the positioning of the Fab regions as well as C_H2, and it is suggested that the hinge has a role in limiting C1q recognition. Notably, an IgG3 mutant in which the complete genetic hinge region of 47 amino acids was replaced by a stretch of 3 alanines followed by a cysteine had a disulfide bond between heavy chains and showed no reduction in ADCML activity [28], emphasizing the importance of the disulfide bridges.

The hybrid molecules produced and purified in this report were positive in ADCML at high and low epitope densities at the same level as the parental IgG3h1-ras molecules (Fig. 7) and were also able to bind C1q and activate complement to the C3 and C5 levels similar to IgG3h1-ras (Fig. 6). Thus, even under very different testing conditions mimicking different epitope densities and distribution on microbial surfaces, the presence of only one C1q binding site is sufficient for full complement activation. This observation is supported by X-ray diffraction analysis, which demonstrated that complete IgG molecules generally have an extremely asymmetric conformation after crystallization, such that only one of the lower hinge proximal C_H2 regions is accessible, as observed for mouse IgG1[37] and IgG2a [38] and human IgG1 [39]. This asymmetry is reflected in the 1:1 stoichiometry found in the interaction between IgGFc and FcγRIII [13]. However, the Fc fragment as such is symmetric, and even so it apparently expresses only one FcγRIII binding site, although both chains seem to be involved [13]. On the other hand, for human FcγRI, only one heavy chain of mouse IgG seems to be involved in the binding [24].

It is reasonable to expect a similar 1:1 stoichiometry for the gC1q:IgG interaction, and our observations with the hybrid molecules would predict such a univalent interaction. The presence of a disulfide bridge between the heavy chains puts the two binding sites for gC1q in close proximity and is probably necessary to make the second gC1q binding site inaccessible once the first is occupied.

If C1q can react at only one side of each IgG molecule, the multivalent interaction between several globular heads of each C1q and IgG would expectedly be under the necessary control. It is essential that accidental activation of the complement cascade is avoided, as this could lead to potentially lethal hyperactive inflammatory responses. Univalency of the IgG molecule with respect to C1q interaction might thus create a necessary threshold for complement activation.

In summary, we show in the present report that a hybrid human IgG1/IgG3 molecule mutated to have only one C1q binding site is able to activate complement efficiently. Human wild-type IgG probably also expose only one C1q binding site. This hypothesis is supported by previous structural analysis of the IgG/FcγR complexes demonstrating exposure of only one FcγR binding site [13]. A theoretical model of IgG/gC1q complexes is also compatible with this notion [16]. We have to wait for more direct experiments involving structural analysis of IgG/C1q complexes or detailed IgG-C1q binding studies in order to verify this hypothesis.

Materials and methods

Construction of mutant IgG1 and IgG3 antibodies

IgG3 with a hinge region characteristic of IgG1 in addition to C131S and R133K mutations was created as described earlier, first denoted OsNP18 [21] and later IgG3 h1 [8]. Amino acids 5–21 from the p21ras proto-oncogene [40] were introduced into the FG loop of the C_H1 domain by *in vitro* mutagenesis as described by Kunkel [41]. A total of 12 nucleotides encoding the FG loop were exchanged by 51 nucleotides, as previously described for other peptides [42]. The mutagenic primer included flanking regions of 20 nucleotides on each side of the core mutagenic nucleotides and is shown in Table 1. The ras5–21 peptide was further mutated from glycine to valine at position 12 using an additional primer (Table 1). The resulting heavy chain, γ3h1-ras, was introduced into the pLNOH2 vector on *Hind*III/*Bam*HI sites [43] to create pLNOH2γ3h1-ras. IgG1 with mutation P329A was constructed by PCR using the wild-type γ1 gene as a template and simultaneously introducing a *Sfi*I restriction site (Fig. 1). Primer sequences are shown in Table 1. 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 and separated by agarose gel electrophoresis. The correct DNA fragments were purified from the gel, ligated using T4 DNA ligase and introduced into the PLNOH2 vector on *Hind*III/*Bam*HI sites [43] to create pLNOH2γ1P329A. Gene sequences were verified by sequencing.

A vector with both complete γ1 and γ3 mutant heavy chain genes as separate expression cassettes was assembled as follows: the pLNOH2γ3h1-ras vector was digested with *Bam*HI, the pLNOH2γ1P329A vector was digested with *Bg*III and *Bam*HI and a fragment containing γ1P329A was isolated and ligated into the *Bam*HI-digested pLNOH2γ3h1-ras vector to create pLNOH2γ3h1-ras/γ1P329A. Both heavy chain genes had exons encoding a murine variable domain with specificity for the hapten NIP. NIP-specific heavy chains can pair with the λ_1 light chains produced by the murine myeloma cell line J558L to form intact antibodies with specificity for NIP. All three pLNOH2 vectors were stably transfected into J558L (a gift from S. L. Morrison, Dept. of Microbiology, Molecular Biology Institute, UCLA) as described [43].

Quantitation of IgG

The amount of NIP-specific IgG, irrespective of subclass, was quantitated by ELISA. Briefly, microtiter plates (Nunc, Maxisorb, Odense, Denmark) were coated with 1 μg/mL $NIP_{15}BSA$. The NIP labeling of BSA has been described [44], and $NIP_{15}BSA$ contains on average 15 NIP groups per BSA molecule. After washing, dilutions (1 μg/mL–20 ng/mL) of an NIP-specific IgG3 standard were added. After incubation for 2 h at 37°C, the plates were washed with PBS containing 0.05% Tween 20 (PBS/T) using a microplate washer (ScanWasher 300, Scatron, Lierbyen, Norway). Then the plates were incubated with a mixture of locally produced, affinity-purified, biotin-labeled sheep anti-human IgGFc [45], streptavidin (Promega, Madison, WI, USA) and locally produced biotin-labeled alkaline phosphatase (biotin-ALP). After incubation for 2 h at 37°C, the plates were washed as described above with PBS/T and developed by addition of 100 μL ALP substrate [1 mg/mL *p*-nitrophenyl phosphate (NPP) in 10% diethanolamine buffer, pH 9.8]. After incubation for 30–60 min at 37°C, the absorbance was recorded at 405 nm using a microplate reader (Thermomax, Molecular Devices, Sunnyvale, CA). Both IgG1 and IgG3 gave linear, parallel dose response curves in the assay.

Detection of hybrid IgG1/IgG3 molecules

Microtiter plates were coated with 2 μg/mL Protein A. Test samples (cell supernatants or eluates from Protein A column) were added in serial dilutions and incubated for 2 h at 37°C. After washing, a mouse monoclonal IgG2b specific for the ras5–21 peptide with mutation G12V (OP38, Calbiochem, Merck Bioscience, Darmstadt, Germany) was added (dilution 1:2000) and the plates incubated for 2 h at 37°C. After another washing step, the plates were incubated with the locally produced biotin-labeled sheep anti-mouse IgGFc [45] mixed with streptavidin and biotin-ALP. The plates were incubated for 2 h at 37°C before a final wash and incubation with NPP. The absorbance was recorded as above.

Table 1. List of primers used to introduce a P329A mutation into the $\gamma1C_H2$ constant domain[a] and to introduce a peptide tag derived from p21ras into the FG loop of $\gamma3C_H1$

	Function	Sequence
Primer 1	Forward primer at 5′-end of γ1 chain containing *HindIII* site	5′-gtggtgatgaagctttctggggcaggccaggcctg[b]
Primer 2	Used to introduce P329A mutation and *Sfi*I site	5′-tttctcgatcccggc**CGC**gagggccttgttggagaccttgcacttc
Primer 3	Used to introduce P329A mutation and *Sfi*I site	5′-ggtctccaacaaggccctc**GCG**gcccccatcgagaaaaccatctc
Primer 4	Reverse primer at 3′-end of γ1 chain containing *Bam*HI site	5′-actactactggatccgacccgctctgcctc
	ras5-21	5′-acacctgcaacgtgaatcac AAACTAGTGGTGGTGGGCGCGGGCGGCGTGGGCAAG TCAGCGCTGACCATCaccaaggtggacaagagagt-3′
	ras V12 mutant	5′- tgggcgcgg**tg**ggcgtgggc-3′

[a] The primer annealing sites are shown in Fig. 1.
[b] The mutation site is shown in capital bold, and the restriction site is underlined.

Affinity chromatography on Protein A

Supernatants from cells transfected with pLNOH2γ3h1-ras/γ1P329A, pLNOH2γ3h1 or pLNOH2γ1P329A genes were subjected to affinity chromatography on a 5 mL HighTrap Protein A column (Amersham Biotech, Uppsala, Sweden). The supernatants (500–1900 mL) were pumped into the column at a rate of 1–1.5 mL/min and eluted with PBS pH 7.3 until base line absorbency was reached. Then, a stepwise elution procedure was carried out using buffers with decreasing pH ranging from 7.3 to 4.0. All eluates were immediately neutralized to pH 7 by addition of 1 M Tris pH 9.0. Each neutralized eluted fraction was concentrated to 500–700 μL volume using ultrafiltration (UH 100/25, Schleicher & Schuell, Dassel, Germany).

Analysis of the Protein A fractions

The fractions were analyzed on a Superdex 200 column (Amersham Biotech) for molecular weight homogeneity using LKB HPLC equipment. Nonreducing gel electrophoresis on SDS-PAGE employing a 10% gel and subsequent immunoblotting were performed by standard procedures using 3% BSA in PBS as a blocking agent. The blots were developed with anti-ras mAb (dilution 1:2000) or biotin-labeled mAb 451D6C3 (dilution 1:4000) for 1.5 h at room temperature. The mAb 451D6C3 was locally produced and shown to have a very similar reaction pattern as Protein A, reacting with IgG1, IgG2 and IgG4 but not with IgG3. mAb 451D6C3 and Protein A efficiently competed with each other in ELISA using IgG1-coated microtiter plates as the target (unpublished data). The blot developed with anti-ras was washed and further developed with biotin-labeled sheep anti-human IgGFc (dilution 1:4000) for 1.5 h at room temperature and washed. Both blots were then incubated with an optimal mixture of biotin-labeled ALP and streptavidin for 1.5 h at room temperature and washed. Finally, the ALP substrate BCIP/NBT (Sigma, B 5655) was added and the blots developed for 10–20 min at room temperature. Parallel to blotting, the polyacrylamide gel was stained with Coomassie Blue using standard procedure.

ADCML assay

The ADCML studies were performed as described previously [31, 44, 46]. Briefly, target ^{51}Cr-labeled sheep red blood cells (SRBC) were allowed to react with NIP-conjugated rabbit anti-SRBC Fab fragments with an average of 4 to 60 NIP groups per Fab fragment. The total amount of NIP introduced onto the surface of 1×10^8 SRBC was 80 ng or 2000 ng; we thus tested the antibodies at low and high antigen concentrations and patchiness on the target cells. The dilution of the added complement serum was 1:30 (1:90 final dilution in the test). Target cells were suspended in a concentration of about 2×10^7 to 3×10^7 cells/mL in the test. Serial dilutions of IgG preparations were then added to the target cell suspension. The antibody concentrations were determined by ELISA using wells coated with $NIP_{15}BSA$ as previously described [30]. The cytotoxic index (CI) was calculated according to the formula: %CI = [(cpm test – cpm spontaneous) / (cpm max – cpm spontaneous)] ×100.

Complement activation measured by ELISA

Complement activation was measured by ELISA using microtiter plates coated with $NIP_{15}BSA$ and developed with anti-C1q [47], anti-C3 or anti-C5. Briefly, the coated ELISA plates were washed with PBS/T and incubated for 1.5 h at 37°C with serial dilutions of antibodies (30–3000 ng/mL) in PBS/T. After washing the plates repeatedly with PBS/T and once with distilled water, aliquots of 100 μL human serum (stored at –70°C) diluted 1:200 in 0.1 M Veronal buffer containing 0.25 mM $CaCl_2$ and 0.8 mM $MgCl_2$ pH 7.2 were added and the plates incubated at 37°C for 30–45 min. After washing with PBS/T, a 1:4000 dilution of rabbit anti-human C5 (Dako, Copenhagen, Denmark) or a 1:10 000 dilution of rabbit anti-human C3c or C1q (both from Dako) in PBS/T was added and the plates incubated for 1.5 h at 37°C. After washing, a mixture of biotin-labeled, locally produced sheep anti-rabbit IgG, biotin-ALP and streptavidin was added. The plates were incubated for 1.5 h at 37°C before washing, final incubation with NPP and reading of the absorbency at 405 nm after various times of incubation.

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