

The following resources related to this article are available online at www.sciencemag.org (this information is current as of November 28, 2009):

Updated information and services, including high-resolution figures, can be found in the online version of this article at:

<http://www.sciencemag.org/cgi/content/full/317/5844/1554>

Supporting Online Material can be found at:

<http://www.sciencemag.org/cgi/content/full/317/5844/1554/DC1>

A list of selected additional articles on the Science Web sites **related to this article** can be found at:

<http://www.sciencemag.org/cgi/content/full/317/5844/1554#related-content>

This article **cites 19 articles**, 9 of which can be accessed for free:

<http://www.sciencemag.org/cgi/content/full/317/5844/1554#otherarticles>

This article has been **cited by** 8 articles hosted by HighWire Press; see:

<http://www.sciencemag.org/cgi/content/full/317/5844/1554#otherarticles>

This article appears in the following **subject collections**:

Immunology

<http://www.sciencemag.org/cgi/collection/immunology>

Information about obtaining **reprints** of this article or about obtaining **permission to reproduce this article** in whole or in part can be found at:

<http://www.sciencemag.org/about/permissions.dtl>

is possibly caused by the missing C-terminal domains that are connected to the outer rim of the torus. Thus, based on a MACPF domain, the main characteristics of the C9 pore can be modeled.

Membrane recognition and pore formation by the complement proteins depend on a sequential assembly of the MAC. The C8 α -MACPF and C8 β -MACPF domains are sufficient for a functional C5b-8 complex, indicating that the flanking N- and C-terminal domains in C8 are not essential for complex formation (17, 27). The flanking domains, however, possibly cover the putative TMHs in soluble C8. Soluble forms of the cell-surface protein CD59 do not bind soluble C8 or C9 (13). CD59 binds residues 365 to 371 of C9 and residues 320 to 415 in C8 α (28), which map to the TMH2 region. Presumably, docking of C8 or C9 onto the MAC reorients the flanking domains exposing the TMHs, which are subsequently “caught in the act” by CD59 present on host cells, and hence the membrane insertion is blocked. In CDCs, membrane insertion only takes place after oligomerization [that is, a large oligomeric prepore is formed on top of the membrane before the membrane is perforated (26)]. C8 α inserts without oligomerization, which is consistent with the hydrophobic character of the putative TMHs. Partial and incomplete pores are observed, when limiting numbers of C9 are available for binding to C5b-8 (11). These data indicate that MAC pore formation is gradual and does not require oligomeric prepores. In this process, C8 plays an important role by binding to the membrane-bound C5b-7 complex, penetrating and destabilizing the membrane, thus readily enabling pore formation by C9.

Perforin, perhaps, acts more like CDCs. Membrane binding by perforin is Ca²⁺-dependent and is mediated by its C-terminal C2 domain (29). The C2 fold is closely related to the fold of the C-terminal d4 domain in CDCs (fig. S5). Notably, the “undcapeptide” membrane-binding site in d4 overlaps with the Ca²⁺-dependent binding site in C2, indicating a common orientation when bound to a membrane. Like in CDCs and C9, the putative TMHs of perforin are amphipathic in character. The amphipathic regions presumably do not penetrate the membrane easily. We argue that unassisted pore formation [as for CDCs, perforin, and in vitro poly(C9)] hence requires formation of a large oligomeric prepore on the membrane to facilitate perforation of the membrane.

The MACPF domain of complement proteins C6 to C9 and perforin is similar to domains d1 and d3 of bacterial CDCs. This finding indicates a possible common evolutionary origin and a common mechanism of membrane insertion. The structural insights could be valuable in the design of therapeutics preventing inappropriate activation of the terminal pathway of complement, as in the case of paroxysmal nocturnal hemoglobinuria and hyperacute rejection of transplanted organs.

References and Notes

- M. J. Walport, *N. Engl. J. Med.* **344**, 1058 (2001).
- M. J. Walport, *N. Engl. J. Med.* **344**, 1140 (2001).
- R. Wurzner, A. Orren, P. J. Lachmann, *Immunodeficiency Rev.* **3**, 123 (1992).
- H. J. Muller-Eberhard, *Annu. Rev. Immunol.* **4**, 503 (1986).
- A. F. Esser, *Toxicology* **87**, 229 (1994).
- J. M. Sodetz, *Curr. Top. Microbiol. Immunol.* **140**, 19 (1989).
- E. W. Steckel, B. E. Welbaum, J. M. Sodetz, *J. Biol. Chem.* **258**, 4318 (1983).
- M. C. Peitsch *et al.*, *Mol. Immunol.* **27**, 589 (1990).
- A. P. Gee, M. D. Boyle, T. Borsos, *J. Immunol.* **124**, 1905 (1980).
- J. Tschopp, H. J. Muller-Eberhard, E. R. Podack, *Nature* **298**, 534 (1982).
- J. Tschopp, *J. Biol. Chem.* **259**, 7857 (1984).
- R. G. DiScipio, T. E. Hugli, *J. Biol. Chem.* **260**, 14802 (1985).
- Y. Huang, F. Qiao, R. Abagyan, S. Hazard, S. Tomlinson, *J. Biol. Chem.* **281**, 27398 (2006).
- E. R. Podack, G. Dennert, *Nature* **302**, 442 (1983).
- G. Dennert, E. R. Podack, *J. Exp. Med.* **157**, 1483 (1983).
- E. R. Podack, H. Hengartner, M. G. Lichtenheld, *Annu. Rev. Immunol.* **9**, 129 (1991).
- D. J. Slade, B. Chiswell, J. M. Sodetz, *Biochemistry* **45**, 5290 (2006).
- J. Rossjohn, S. C. Feil, W. J. McKinstry, R. K. Tweten, M. W. Parker, *Cell* **89**, 685 (1997).
- G. Polekhina, K. S. Giddings, R. K. Tweten, M. W. Parker, *Proc. Natl. Acad. Sci. U.S.A.* **102**, 600 (2005).
- S. J. Tilley, H. R. Saibil, *Curr. Opin. Struct. Biol.* **16**, 230 (2006).
- R. K. Tweten, *Infect. Immun.* **73**, 6199 (2005).
- K. S. Giddings, J. Zhao, P. J. Sims, R. K. Tweten, *Nat. Struct. Mol. Biol.* **11**, 1173 (2004).
- M. W. Parker, S. C. Feil, *Prog. Biophys. Mol. Biol.* **88**, 91 (2005).
- L. A. Shepard *et al.*, *Biochemistry* **37**, 14563 (1998).
- S. Shatursky *et al.*, *Cell* **99**, 293 (1999).
- S. J. Tilley, E. V. Orlova, R. J. Gilbert, P. W. Andrew, H. R. Saibil, *Cell* **121**, 247 (2005).
- C. L. Brannen, J. M. Sodetz, *Mol. Immunol.* **44**, 960 (2007).
- D. H. Lockert *et al.*, *J. Biol. Chem.* **270**, 19723 (1995).
- I. Voskoboinik *et al.*, *J. Biol. Chem.* **280**, 8426 (2005).
- W. L. Delano, The PyMOL Molecular Graphics System (DeLano Scientific, San Carlos, CA, 2002), <http://pymol.sourceforge.net>.
- Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
- We thank T. H. C. Brondijk, B. J. C. Janssen, F. J. Milder, and L. Rutten for assistance, M. R. Daha, E. G. Huizinga and J. A. G. van Strijp for critically reading the manuscript. We thank the European Synchrotron Radiation Facility for providing synchrotron radiation facilities and the beamline scientists at ID-29 for their help with data collection. This work was supported by a “Pionier” grant (P.G.) of the Council for Chemical Sciences of the Netherlands Organization for Scientific Research. Coordinates and structure factors have been deposited in the Protein Data Bank (PDB) (www.rcsb.org) under accession code 2QQH.

Supporting Online Material

www.sciencemag.org/cgi/content/full/317/5844/1552/DC1

Materials and Methods

Figs. S1 to S5

Table S1

References

27 June 2007; accepted 3 August 2007

10.1126/science.1147103

Anti-Inflammatory Activity of Human IgG4 Antibodies by Dynamic Fab Arm Exchange

Marijn van der Neut Kolfshoten,¹ Janine Schuurman,² Mario Losen,³ Wim K. Bleeker,² Pilar Martínez-Martínez,³ Ellen Vermeulen,¹ Tamara H. den Bleker,¹ Luus Wiegman,² Tom Vink,² Lucien A. Aarden,¹ Marc H. De Baets,^{3,4} Jan G.J. van de Winkel,^{2,5} Rob C. Aalberse,^{1*} Paul W. H. I. Parren^{2*}

Antibodies play a central role in immunity by forming an interface with the innate immune system and, typically, mediate proinflammatory activity. We describe a novel posttranslational modification that leads to anti-inflammatory activity of antibodies of immunoglobulin G, isotype 4 (IgG4). IgG4 antibodies are dynamic molecules that exchange Fab arms by swapping a heavy chain and attached light chain (half-molecule) with a heavy-light chain pair from another molecule, which results in bispecific antibodies. Mutagenesis studies revealed that the third constant domain is critical for this activity. The impact of IgG4 Fab arm exchange was confirmed in vivo in a rhesus monkey model with experimental autoimmune myasthenia gravis. IgG4 Fab arm exchange is suggested to be an important biological mechanism that provides the basis for the anti-inflammatory activity attributed to IgG4 antibodies.

In the classic paradigm, immunoglobulins present products of clonal B cell populations, each producing antibodies recognizing a single antigen specificity (1, 2). Human immunoglobulin G (IgG) antibodies exist in four subclasses with distinct structural and functional properties. IgGs are composed of two heavy chain–light chain pairs (half-molecules), which are connected via inter-heavy chain disulfide bonds situated in the hinge region, as well as by noncovalent bonds mostly situated between the

third constant (C_H3) domains (3). Stability of the inter-heavy chain disulfide bonds varies between subclasses, and for IgG4 in particular, intra-heavy chain disulfide bonds may be formed instead (4).

IgG4 antibodies differ functionally from other IgG subclasses in their anti-inflammatory activity, which includes a poor ability to induce complement and cell activation because of low affinity for C1q (the q fragment of the first component of complement) and Fc receptors (5, 6).

Consequently, IgG4 has become the preferred subclass for immunotherapy, in which recruitment of host effector function is undesirable. Another, more poorly understood property of blood-derived IgG4 is its inability to cross-link identical antigens, which is referred to as “functional monovalency” (7, 8). In contrast, cross-linking of nonidentical antigens (bispecificity) has been observed under certain conditions (9). It has been postulated that these observations might be explained by the exchange of half-molecules between distinct IgG4 molecules (9–11). This hypothesis, however, has not been widely accepted, as this exchange previously could not be confirmed using recombinant IgG4 antibodies (9). Here we show, using well-characterized monoclonal antibodies, that Fab arm exchange represents a general characteristic of IgG4.

We explored this issue using blood-derived and recombinant IgG4 antibodies against the major birch pollen (Betv1) and cat (Feld1) allergens (12). Serum from an allergic patient with high IgG4 titers against both antigens contained bispecific antibodies that cross-linked Betv1 and Feld1, whereas such reactivity was not detected in control sera (fig. S1). This peculiar bispecific reactivity of blood-derived IgG4 could not be reproduced by simple mixing in vitro (fig. S2), as previously observed for other antigens (13). To investigate whether bispecificity is the result of in vivo molecular processing, we injected an equal mixture of recombinant IgG4-Betv1 and IgG4-Feld1 or corresponding IgG1 controls into immunodeficient mice. Blood samples were drawn, after which we measured Feld1/Betv1-bispecific IgG (Fig. 1A). Interestingly, bispecific antibodies appeared in the blood of mice injected with IgG4, but not IgG1, mixtures. A stochastic Fab arm exchange between equal amounts of IgG4-Betv1 and IgG4-Feld1 would be consistent with approximately half of the IgG4 molecules acquiring bispecificity (14). To demonstrate the plasticity of this process, we injected an IgG4 mixture including a 20-fold excess of an additional IgG4 with irrelevant specificity, which almost abrogated Feld1/Betv1-bispecific reactivity (Fig. 1A). This inhibition is explained by the generation of bispecific IgG4 not detected in our Feld1-Betv1 cross-linking assay. If the observed bispecificity resulted from Fab arm exchange, then the increase in bispecific activity should be accompanied by an equivalent loss of mono-

specific cross-linking activity (15), which was indeed observed (fig. S3).

IgG molecules are protected from catabolism by the neonatal Fc receptor (FcRn) by an intracellular recycling route (16). However, FcRn was not required for Fab arm exchange because no differences in the generation of bispecific IgG4 were observed between wild-type and FcRn-deficient mice (fig. S4). This suggests that Fab arm exchange may occur in the extracellular milieu.

The molecular mechanisms for Fab arm exchange were studied by defining in vitro conditions. We found that bispecific IgG4, but not IgG1, molecules were generated on mixing with isolated blood cells, but not with human serum. Bispecific IgG4 molecules were generated in the presence of human erythrocytes with similar kinetics as observed in vivo (Fig. 2A). A low-molecular-weight compound from erythrocyte lysates appeared essential for the exchange reaction (table S1). This observation, in combination with the requirement for breaking inter-heavy chain disulfide bonds, prompted us to test whether bispecific IgG4 was generated in the presence of small reducing agents such as reduced glutathione (GSH). Notably, the addition of GSH or other reducing agents to a mixture of purified IgG4, but not IgG1, was sufficient to induce Fab arm exchange (Fig. 2B). The reaction in the presence of GSH occurred more efficiently at physiological than at low temperature (fig. S5).

We extended our analyses to fully human monoclonal antibodies (HumAbs) with a matched set of well-characterized recombinant human IgG1 and IgG4 antibodies directed against CD20 antigen and epidermal growth factor receptor (EGFR) [HumAbs 7D8 (17) and 2F8 (18), respectively]. Bispecificity was evaluated with a sandwich enzyme-linked immunosorbent assay (ELISA) consisting of recombinant EGFR immobilized on the solid phase and an anti-idiotype antibody against HumAb 7D8 as a detecting reagent. The kinetics of bispecific antibody formation against EGFR/CD20, which occurred only for the IgG4 mixture and only in the presence of GSH, is shown in Fig. 2C. The molecular requirements for Fab arm exchange were studied by mutagenesis. Unexpectedly, our experiments suggest that the C_H3 domain and not the core hinge is dominantly involved in the reaction. Thus, exchanging C_H3 domains between IgG1 and IgG4 activated Fab arm exchange for IgG1 and abrogated activity for IgG4 (Fig. 2D). In contrast, replacing the IgG1 core hinge sequence with the IgG4 sequence alone [by replacing Pro²²⁸ with Ser (P228S)] had no effect, and the IgG4 C_H3 sequence was additionally required for full Fab arm exchange activity by the IgG1 mutant (Fig. 2D).

To conclusively demonstrate that Fab arm exchange is indeed the result of an intermolecular exchange reaction, we analyzed the molecules generated using size-exclusion chromatography and mass spectrometry (MS). First, we performed size-exclusion analyses of plasma from two of the mice injected with an IgG4 antibody

mixture. Bispecific antibodies eluted at the expected position for monomeric IgG, which ruled out the possibility that the observed reactivity was due to aggregation (fig. S6). Second, we incubated a mixture of IgG4-EGFR and IgG4-CD20 in the absence or presence of GSH, followed by mass spectrometry [electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS)]. Figure 2E shows that the molecular masses of IgG4-CD20 (145.5 kD) and IgG4-EGFR (145.9 kD) remained unchanged in the absence of GSH. In the presence of GSH (Fig. 2F), however, a new peak with an intermediate mass appeared (145.7 kD). The novel mass corresponded to the expected mass of the bispecific antibody against EGFR/CD20 detected in Fig. 2C. Moreover, from the peak heights of the MS spectra it was estimated that the bispecific anti-

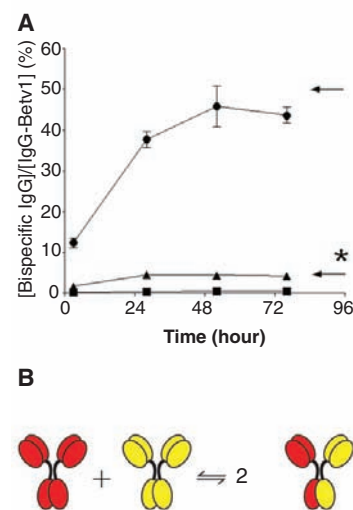
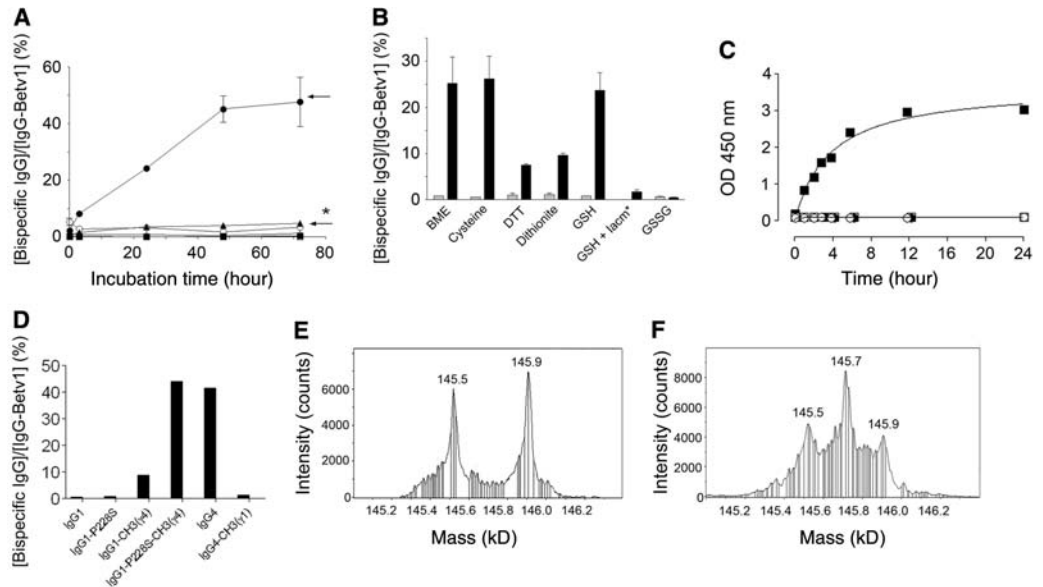


Fig. 1. Bispecific human IgG4 molecules are generated in vivo. **(A)** Groups ($n = 5$) of nude mice were injected with antibody mixtures: IgG1-Betv1/IgG1-Feld1 (squares), IgG4-Betv1/IgG4-Feld1 (circles), or IgG4-Betv1/IgG4-Feld1 + irrelevant recombinant IgG4 (IgG4-EGFR; triangles) (test antibodies, 100 μ g each per mouse; irrelevant IgG4, 2000 μ g per mouse). The generation of bispecific antibodies was followed by assessing Betv1 and Feld1 bispecific reactivity, which was expressed as a percentage relative to the total IgG-Betv1 concentration. The top arrow indicates the expected level of bispecific reactivity (50%) in mice receiving an equal amount of IgG4-Betv1 and IgG4-Feld1 (14); the bottom arrow with asterisk indicates the expected level of bispecific reactivity (4%) in the presence of excess irrelevant IgG4. Error bars represent SEM. **(B)** IgG4 Fab arm exchange occurs by the exchange of a heavy chain–light chain pair (half-molecule) of one IgG4 molecule with that of another IgG4 molecule. The IgG4 molecule may thereby acquire two distinct Fab arms and become bispecific. The Fc structure remains essentially unchanged apart from potential changes due to differences in glycosylation or allotype. Fab arm exchange is proposed to be stochastic and dynamic.

¹Sanquin Research—AMC Landsteiner Laboratory, Department of Immunopathology, Plesmanlaan 125, 1066 CX Amsterdam, the Netherlands. ²Genmab, Yalelaan 60, 3584 CM Utrecht, the Netherlands. ³Department of Neuroscience, Institute of Mental Health and Neuroscience, University of Maastricht, Maastricht, the Netherlands. ⁴Neuroimmunology Group, Biomedical Research Institute (BIOMED) Hasselt University, Diepenbeek, Belgium. ⁵Immunotherapy Laboratory, Department of Immunology, University Medical Center, Utrecht, the Netherlands.

*To whom correspondence should be addressed. E-mail: r.aalberse@sanquin.nl (R.C.A.); p.parren@genmab.com (P.W.H.I.P.)

Fig. 2. Induction of IgG4 Fab arm exchange in vitro. **(A)** Incubation of IgG4-Betv1/IgG4-Feld1 mixtures (circles) with human erythrocytes (closed symbols) at 37°C resulted in generation of bispecific antibodies; no bispecificity was generated in IgG1 mixtures (squares). As controls, an excess of irrelevant IgG4 was added (triangles) or antibody mixtures were incubated without erythrocytes (open symbols). Error bars represent range of duplicate measurements. **(B)** IgG4-Betv1/IgG4-Feld1 mixtures (black bars) were incubated for 24 hours at 37°C in the presence of different reducing agents, and the generation of bispecific IgG was determined. IgG1 antibody mixtures were used as controls (gray bars). Error bars represent SEM calculated from three measurements. **(C)** IgG4-CD20/IgG4-EGFr mixtures (squares) were incubated at 37°C with (closed symbols) or without (open symbols) 0.5 mM GSH. The formation of bispecific antibodies was measured in ELISA. OD₄₅₀, optical density (absorbance) at 450 nm. Mixtures of IgG1 antibodies were used as controls (circles). Data are representative of three experiments. **(D)** IgG1 and IgG4 mutants were prepared for the Betv1 and Feld1 antibodies. P228S represents a point mutation that introduces the IgG4 core hinge sequence into IgG1. C_H3(γ1) and C_H3(γ4) represents domain swap mutations in which the original third



constant domain is replaced with the IgG1 or IgG4 domain, respectively. Fab arm exchange between corresponding mutants is shown. **(E)** IgG4-EGFR/IgG4-CD20 mixtures were incubated for 24 hours in the absence **(E)** or presence **(F)** of 0.5 mM GSH, after which the antibodies were deglycosylated with peptide *N*-glycosidase F, and the molecular masses of the resulting antibodies were determined by ESI-TOF mass spectrometry. Deconvoluted ESI-TOF spectra are shown. Data are representative of two experiments.

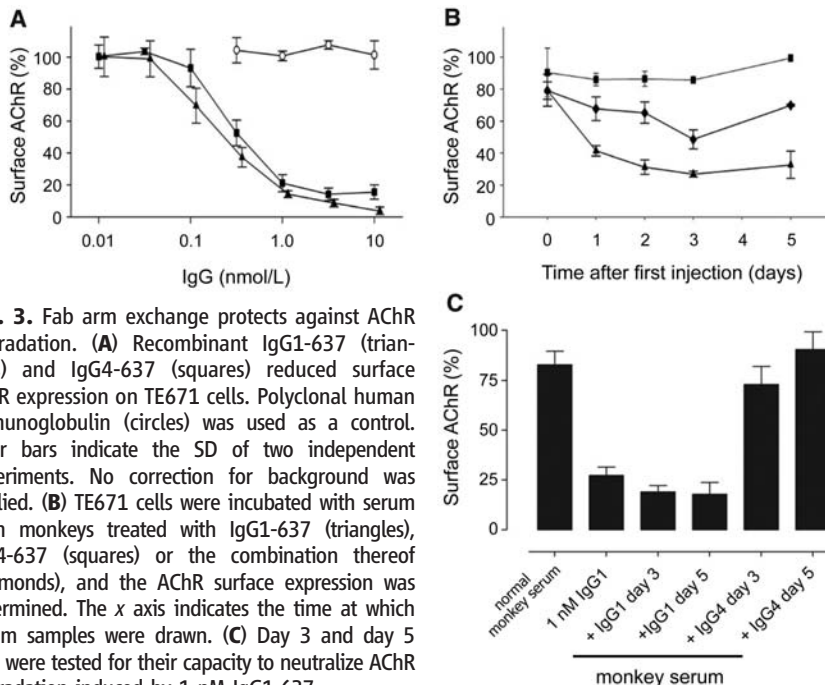


Fig. 3. Fab arm exchange protects against AChR degradation. **(A)** Recombinant IgG1-637 (triangles) and IgG4-637 (squares) reduced surface AChR expression on TE671 cells. Polyclonal human immunoglobulin (circles) was used as a control. Error bars indicate the SD of two independent experiments. No correction for background was applied. **(B)** TE671 cells were incubated with serum from monkeys treated with IgG1-637 (triangles), IgG4-637 (squares) or the combination thereof (diamonds), and the AChR surface expression was determined. The *x* axis indicates the time at which serum samples were drawn. **(C)** Day 3 and day 5 sera were tested for their capacity to neutralize AChR degradation induced by 1 nM IgG1-637.

body represented 50% of the total antibody mass in the mixture, which indicated a stochastic exchange (Fig. 1B).

We investigated how Fab arm exchange contributes to the anti-inflammatory properties of IgG4 in an animal model in which antibody-mediated cross-linking plays an important role in disease pathogenesis. Experimental autoimmune myasthenia gravis (MG), in which autoanti-

bodies against acetylcholine receptor (AChR) induce muscle weakness, represents such a model (19). The human AChR-specific antibody IgG1-637 is a patient-derived antibody (20) that induces AChR degradation as can be monitored in vitro using rhabdomyosarcoma TE671 cells. Both IgG1-637 and IgG4-637 antibodies reduced TE671 cell surface AChR levels to the same extent (Fig. 3A), which indicated that these anti-

Table 1. IgG4-637 protects monkeys from IgG1-637-induced myasthenia gravis. Total cumulative dose is the sum of antibodies injected as three doses on three consecutive days. Clinical symptoms started between day 1 and 5 post injection and lasted up to 7 days. At the peak of the disease, animals were hypoactive; could not climb because of weakness of the limbs, hands, and feet; and had difficulty eating.

Antibody	Total cumulative dose (mg/kg)	Animals (n)	Clinical symptoms
IgG1-637	1.5	1	No (0/1)
IgG1-637	3	1	No (0/1)
IgG1-637	15	1	Yes (1/1)
IgG1-637	5	4	Yes (4/4)
IgG4-637	15	2	No (2/2)
IgG1-637	5	5	No (5/5)
IgG4-637	15	5	No (5/5)

bodies reacted similarly in vitro. IgG1-637 is a pathogenic antibody, and it induced MG disease dose-dependently in rhesus monkeys (Table 1). The IgG4-637 antibody, in contrast, was not pathogenic (Table 1). To assess whether IgG4-637 might protect against IgG1-637-induced disease, we injected IgG4-637, followed by challenge with a pathogenic dose of the IgG1 antibody. All animals were protected (*n* = 5), which represents a significant difference compared with treatment with IgG1-637 alone (*P* < 0.01; two-tailed Fisher's exact test) (Table 1). Notably, protection against MG disease correlated with an in vivo decrease of AChR cross-linking activity of the injected IgG4 antibody. Thus, serum

from an IgG1-637-treated monkey induced strong AChR degradation, in contrast to serum from a monkey injected with the IgG4 antibody (Fig. 3B). The latter serum, moreover, prevented AChR degradation by IgG1-637 in an in vitro mixing experiment (Fig. 3C). Finally, serum from an animal treated with both antibodies induced only moderate degradation. The IgG4-637 antibody, which induced AChR degradation before injection (Fig. 3A), apparently acquired non-cross-linking and protective activity in vivo.

Antibody function is regulated by posttranslational modifications. Fucosylation in the Fc domain, for example, affects immune cell activation by modulating affinity for Fc receptors (21). Sialylation has been shown to endow IgG with anti-inflammatory activity (22), but that cannot explain our findings, as none of the antibodies used contained sialic acid. Dynamic Fab arm exchange therefore represents a novel type of posttranslational modification, which serves as an additional mechanism for generating anti-inflammatory activity. The mechanism by which IgG4 Fab arm exchange occurs in vivo likely requires the reducing environment in blood or at cell surfaces to facilitate the breaking of inter-heavy chain disulfide bonds located in the hinge region. Indeed, the addition of reducing compounds, such as GSH, to purified IgG4 alone was sufficient to induce in vitro Fab arm exchange. GSH, present in all cell types, may well perform this role in vivo, and so additional cofactors, chaperones, or receptors, as hypothesized for PDI and FcRn previously (10), may therefore not be essential. An important second antibody heavy chain interface is located between the C_H3 domains, which we show to be critically involved in Fab arm exchange. Elucidating the contribution of specific C_H3-domain amino acid contacts to the mechanism of this reaction requires further investigation.

We show that IgG4 molecules acquire two distinct Fab arms by Fab arm exchange, which, when derived from polyclonal plasma IgG4, are (usually) directed against unrelated antigens. Fab arm exchange, furthermore, is dynamic, and combinations of certain specific Fab arms are therefore only expected to exist transiently. IgG4 molecules thereby lose their ability to cross-link antigen and to form immune complexes under most conditions. IgG4, often induced by chronic antigen stimulation, then may interfere with immune complex formation by other antibody isotypes and may dampen inflammatory reactions. In specific immunotherapy with allergen in allergic rhinitis, for example, increases in allergen-specific IgG4 levels indeed correlate with clinical responses (7, 23). A first proof of concept using monoclonal antibodies indicates that the formation of non-cross-linking IgG4 antibodies in vivo provided protection against a pathogenic antibody in experimental autoimmune MG.

Our results have an impact on immunotherapy with IgG4 monoclonal antibodies. The in vivo instability and dynamics of IgG4

introduce unpredictability, which is undesirable for human immunotherapy. Future studies should address the contribution of IgG4 Fab arm exchange to in vivo activity of therapeutic IgG4 monoclonal antibodies.

In summary, antibodies of the IgG4 isotype are shown to be dynamic molecules, undergoing Fab arm exchange in vivo and in vitro. The ability to engage in Fab arm exchange appears to be an inherent feature of IgG4 that involves the third constant domain in addition to the hinge region and that only requires a reducing environment to be activated. This novel protein modification challenges the commonly accepted one antibody-one antigen paradigm and redefines our thinking about the role of IgG4 in antibody-mediated immunity and the application of IgG4 monoclonal antibodies to immunotherapy.

References and Notes

1. F. M. Burnet, *Aust. J. Sci.* **20**, 67 (1957).
2. G. M. Edelman *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **63**, 78 (1969).
3. E. O. Saphire *et al.*, *J. Mol. Biol.* **319**, 9 (2002).
4. J. Schuurman, G. J. Perdok, A. D. Gorter, R. C. Aalberse, *Mol. Immunol.* **38**, 1 (2001).
5. J. S. van der Zee, P. van Swieten, R. C. Aalberse, *Clin. Exp. Immunol.* **64**, 415 (1986).
6. R. Jefferis, in *The Human IgG Subclasses*, F. Shakib, Ed. (Pergamon Press, Oxford, 1990), pp. 15–30.
7. J. S. van der Zee, P. van Swieten, R. C. Aalberse, *J. Immunol.* **137**, 3566 (1986).
8. R. A. Margni, R. A. Binaghi, *Annu. Rev. Immunol.* **6**, 535 (1988).
9. J. Schuurman *et al.*, *Immunology* **97**, 693 (1999).
10. R. C. Aalberse, J. Schuurman, *Immunology* **105**, 9 (2002).
11. B. J. Scallon *et al.*, *J. Immunother.* **29**, 351 (2006).
12. Materials and methods are available as supporting material on Science Online.
13. R. C. Aalberse, J. Schuurman, R. van Ree, *Int. Arch. Allergy Immunol.* **118**, 187 (1999).
14. For calculations, see supporting online material. A maximum of 50% of IgG4-Betv1 half-molecules are

- incorporated in bispecific antibodies if equal amounts of IgG4 are mixed and if exchange is stochastic. The term stochastic indicates that exchange occurs without preference and results in complete mixing at equilibrium.
15. We prefer to describe the classical bivalency of IgG as monospecific cross-linking activity.
 16. R. P. Junghans, C. L. Anderson, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 5512 (1996).
 17. J. L. Teeling *et al.*, *Blood* **104**, 1793 (2004).
 18. W. K. Bleeker *et al.*, *J. Immunol.* **173**, 4699 (2004).
 19. M. Losen *et al.*, *Brain* **128**, 2327 (2005).
 20. Y. F. Graus *et al.*, *J. Immunol.* **158**, 1919 (1997).
 21. R. L. Shields *et al.*, *J. Biol. Chem.* **277**, 26733 (2002).
 22. Y. Kaneko, F. Nimmerjahn, J. V. Ravetch, *Science* **313**, 670 (2006).
 23. A. Nanda *et al.*, *J. Allergy Clin. Immunol.* **114**, 1339 (2004).
 24. We thank P. de Heer, J. Scheik, A. Ortiz Buijsse, A. Verwilligen, and G. Perdok for technical assistance; H. de Vrieze for providing patient sera; J. Werkman for his help with the generation of the IgG mutants; K.V. Toyka, H. Brok, and B. 't Hart for their help with the rhesus monkey model; J. Bakker for graphics; and S. Ruuls for critically reviewing the manuscript. This work was supported by grants from the Prinses Beatrix Fonds, L'Association Française contre les Myopathies, a Marie Curie Fellowship, project grant QL63-CT-2001-00225 of the European Community program "quality of life and management of living resources," the European Union Sixth Framework Programme (FP6) MYASTAID LSMM-CT-2006-037833, Joghem van Loghem Foundation, and Genmab. Genmab is an international biotechnology company that creates and develops human antibodies for immunotherapy. Genmab has partnered antibody products with Amgen, Roche, and GlaxoSmithKline. Authors specified below have a financial interest in Genmab. J.S., W.K.B., L.W., T.V., J.G.J.W., and P.W.H.I.P. have stock and/or warrants; L.A.A. and R.C.A. received consulting fees.

Supporting Online Material

www.sciencemag.org/cgi/content/full/317/5844/1554/DC1
Materials and Methods
Figs. S1 to S6
Table S1
References

3 May 2007; accepted 8 August 2007
10.1126/science.1144603

Functional Architecture and Evolution of Transcriptional Elements That Drive Gene Coexpression

Christopher D. Brown,¹ David S. Johnson,¹ Arend Sidow^{1,2,*}

Transcriptional coexpression of interacting gene products is required for complex molecular processes; however, the function and evolution of cis-regulatory elements that orchestrate coexpression remain largely unexplored. We mutagenized 19 regulatory elements that drive coexpression of *Ciona* muscle genes and obtained quantitative estimates of the cis-regulatory activity of the 77 motifs that comprise these elements. We found that individual motif activity ranges broadly within and among elements, and among different instantiations of the same motif type. The activity of orthologous motifs is strongly constrained, although motif arrangement, type, and activity vary greatly among the elements of different co-regulated genes. Thus, the syntactical rules governing this regulatory function are flexible but become highly constrained evolutionarily once they are established in a particular element.

Gene products that are involved in the same molecular process must be coordinately expressed. Transcriptional coexpression is achieved by regulatory proteins and

their target cis-regulatory elements that promote gene transcription in overlapping spatiotemporal distributions (1–7). The function of a cis-element is encoded in its molecular architecture: a nucle-