Human IgG4 Binds to IgG4 and Conformationally Altered IgG1 via Fc-Fc Interactions

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The Fc fragment of IgG4 can interact with the Fc fragment of another IgG molecule. This interaction is a confounding factor when measuring IgG4 rheumatoid factor levels. Recently, we demonstrated that half-molecules of IgG4 can exchange to form a bispecific Ab. We expected these two phenomena to be related and investigated the physicochemical aspects of IgG4 Fc-Fc interactions. We found that IgG4 is >99% monomeric by size-exclusion chromatography; therefore, IgG4 Fc-Fc interactions in the fluid phase (if any) would be short-lived. However, ^{125}I-labeled IgG4 does bind to IgG1 and IgG4 coupled to a solid phase. By contrast, IgG1 does not bind to coupled IgG4. Furthermore, conditions that induce partial unfolding/dissociation of the CH3 domains enhance IgG4 Fc binding, suggesting that Fc binding is primarily CH3 mediated. IgG4 slowly associates with both IgG4 and IgG1 coupled to a biosensor chip. Remarkably, subsequent dissociation was much faster for IgG4 than for IgG1. Moreover, after binding of an IgG4 mAb to Sepharose-coupled Ag, we observed additional binding of IgG4 with irrelevant specificity, whereas similar binding was not observed with Ag-bound IgG1. We propose that the IgG4-IgG4 Fc interaction resembles an intermediate of the Fab-arm (half-molecule) exchange reaction that is stabilized because one of the IgG4 molecules is coupled to a solid phase. By contrast, IgG4 Fc recognizes IgG1 only after a conformational change that renders CH3(IgG1) accessible to an interaction with the CH3(IgG4). Such Fc interactions may enhance Ag binding of IgG4 in vivo. *The Journal of Immunology,* **2009, 182: 4275–4281.**

Human IgG4 combines several properties not found for the other subclasses (1). In particular, as illustrated in Fig. 1, IgG4 can exchange half-molecules (H + L chain) with other IgG4 molecules, combining two different specificities in a single Ab molecule (2). This process was named "Fab-arm exchange," which emphasizes the consequences of the half-molecule exchange reaction. In vivo, the large number of possible recombinations usually rules out the buildup of significant concentrations of any particular type of bispecific IgG4. On the other hand, the valency for each specificity changes from 2 to 1 during the exchange. The result is an IgG4 molecule that is effectively monovalent. It is able to act as blocking Ab, but is incapable of forming large immune complexes (3, 4). The mechanism of exchange, which occurs both in vivo and, in the presence of glutathione, in vitro, is not fully understood, but may well involve a preassociation step of two intact, four-chain IgG4 molecules.

An interaction that resembles such a preassociation, binding of IgG4 to IgG1, is described in the context of rheumatoid arthritis (RA).[2] Many patients with RA produce autoantibodies directed against the Fc portion of IgG (rheumatoid factors, RF). These RF form immune complexes in the joint that may contribute to chronic inflammation. A significant proportion of IgG RF activity was attributable to IgG4 (5). Interestingly, although generally RF activity involves variable domain-mediated binding to IgG-Fc, it is reported that IgG4 possesses IgG1-Fc binding activity that is located in the constant domains (6). The main contribution to this type of binding was found to arise from the Fc part of the IgG4 molecule. Apart from giving rise to, possibly false, high RF titers, this Fc-mediated binding of IgG4 to IgG1 Abs may result in overestimated values for IgG4 in binding assays involving catching Abs, depending on the animal source. Elevated levels of IgG4 are associated with other RA-related autoantibodies as well (7).

We characterized the physicochemical aspects of IgG4 binding to IgG using several solid-phase assays (RIA, ELISA, surface plasmon resonance (SPR)) as well as a fluid-phase assay (high-performance size-exclusion chromatography (HP-SEC)). We were interested to see whether Fc-Fc interactions can also occur between IgG4 molecules. In a cross-linking assay that we used previously to demonstrate bispecific IgG4 molecules, background binding of monospecific IgG4 was found (which was inhibited by i.v. IgG), presumably due to IgG4-IgG4 Fc interactions. Using a simple, direct radioimmunoassay, we were indeed able to demonstrate binding of IgG4 to IgG4 and to compare these interactions with IgG4 to IgG1. Surprisingly, we could demonstrate the interaction only using a solid-phase binding assay. The present results suggest that rather than a preassociation the Fc binding resembles an intermediate of the half-molecule exchange reaction of IgG4.

Materials and Methods

Recombinant birch allergen Bet v 1 was provided by Dr. M. Wallner (University of Salzburg). Recombinant chimeric IgG1 and IgG4 Abs against Bet v 1 and Fel d 1 were produced as described previously (2). Other monoclonal chimeric or human(ized) IgG1 and IgG4 Abs used are natalizumab (Tysabri; Biogen Idec), and adalimumab (Humira; Abbott Laboratories).

Fc fragments of IgG1 (adalimumab) or IgG4 (natalizumab) were obtained by papain digestion. Ten milligrams of mAb was digested with 0.1 mg of preactivated papain (2.1 mg/ml, 1 mM DTT, 30 min, 37°C) for 3 h

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[2] Abbreviations used in this paper: RA, rheumatoid arthritis; RF, rheumatoid factor; HP-SEC, high-performance size-exclusion chromatography; CNBr, cyanogen bromide; GSH, glutathione; RU, response unit; SPR, surface plasma resonance.

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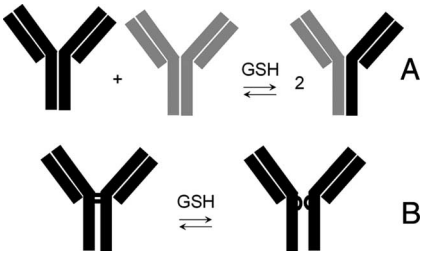

FIGURE 1. *A*, In the GSH-mediated exchange reaction, one-half of an IgG4 molecule (=H chain + L chain) recombines with a half-molecule of another IgG4 to form a bispecific IgG4 molecule. *B*, The hinge of IgG4 contains four cysteines that can form either two interchain or intrachain disulfide bridges.

at 37°C in 0.5 ml of PBS (pH 7.4)/3 mM EDTA. Fab and Fc were separated from undigested material by size-exclusion chromatography (Superdex Hi-Load 16/60 column; GE Healthcare) and Fc was separated from Fab on a Mono Q column (10 mM Tris (pH 8.0), linear gradient from 0 to 0.35 M NaCl).

Recombinant IgG4 Fc was produced under serum-free conditions (FreeStyle 293 Expression Medium from Invitrogen) by transfecting pFUSE-hIgG4-Fc2 expressing vector (InvivoGen) in HEK-293F cells using 293fectin according to the manufacturer's instructions (Invitrogen). Cell culture supernatants were centrifuged for 15 min at 1700 × *g*, followed by loading on a protein G column (Protein G 4 fast flow; GE Healthcare) and elution of the Fc with 0.1 M glycine (pH 2.5). The eluate was neutralized immediately with 2 M Tris-HCl (pH 9) and dialyzed overnight to PBS. After dialysis, samples were stored at −20°C.

Labeling

Anti-Fel d 1 IgG4, anti-Bet v 1 IgG4, and recombinant IgG4 Fc were labeled by the ^{125}I chloramine-T method as described previously (8).

Sepharose-binding assay

In this binding assay, IgG (anti-Fel d 1 IgG1, anti-Fel d 1 IgG4, anti-Bet v 1 IgG4, infliximab, adalimumab, or omalizumab) was coupled to CNBr-activated Sepharose (Amersham Biosciences). Unless stated otherwise in the text, 250 μl of a 2-mg/ml Sepharose suspension was incubated overnight with ^{125}I-labeled IgG4, IgG4 Fc, or IgG1 in a total volume of 750 μl of PBS-AT (PBS, pH 7.4, supplemented with 0.3% BSA/0.1% Tween 20, 10 mM EDTA, and 0.05% (w/v) NaN_3) on a vertical rotor at room temperature. After washing five times, binding was measured. Percentages of binding are expressed as the amount of radioactivity bound relative to the amount of radioactivity added.

Binding at different pH was investigated similarly using pH-adjusted buffer. Preincubation of adalimumab-Sepharose at different pH was conducted in 10 mM acetate or phosphate buffer with a pH between 1 and 7.4. After 30 min, samples were neutralized by washing five times with PBS-AT, and binding of ^{125}I-labeled IgG4 Fc was determined as described above.

Polystyrene-binding assay (microtiter plate)

For this assay, a microtiter plate (Greiner bio-one) was coated for 1 h with 1 μg/ml (100 μl/well) of IgG in PBS at 37°C. The plate was washed three times with PBS/0.05% Tween 20 (PBST) and blocked with PBS/0.1% BSA for 1 h at 37°C. After washing three times, ^{125}I-labeled anti-Fel d 1 IgG4 or anti-Bet v 1 IgG4 was added and incubated overnight at room temperature. The plate was washed three times and the coated material was transferred to test tubes using 2 × 200 μl of 0.1 M NaOH. Percentages of binding are measured (expressed as the amount of radioactivity bound relative to the amount of radioactivity added).

Indirect Sepharose-binding assay

Fel d 1 and Bet v 1 allergens were coupled to CNBr-activated Sepharose (0.16–1.25 mg/100 mg). In the case of Fel d 1, 50 μl of an anti-Fel d 1 IgG4 solution (0.02–20 μg/ml) and 250 μl of a 2-mg/ml Sepharose suspension in a total volume of 750 μl of PBS-AT were incubated overnight on a vertical rotor at room temperature. After washing five times, Sepharose-bound Ab was allowed to react with ^{125}I-labeled anti-Bet v 1 IgG4 overnight, washed five times with PBS-AT, and the binding was measured. Alternatively, the Sepharose was incubated with anti-Fel d 1 IgG4 along with anti-Bet v 1 IgG1 or IgG4. After washing five times, ^{125}I-labeled Bet v 1 was added and incubated overnight. After washing five times, binding was measured. Both protocols were also conducted vice versa, i.e., with Bet v 1 Sepharose.

SPR measurements

SPR measurements were performed using a Biacore 3000 instrument. All ligands (adalimumab Fc, natalizumab Fc) were immobilized at a concentration of 27 nM in 10 mM sodium acetate (pH 5.0) on a CM5 sensor chip using *N*-hydrosuccinimide/1-ethyl-3-(3dimethylaminopropyl)carbodiimide hydrochloride at a flow rate of 5 μl/min. Similarly, transferrin, with a pI close to that of IgG, was coupled as a negative control. Binding of IgG1 Fc and IgG4 Fc (0.04–1.23 μM in PBS with 0.01% Tween 20, PBS-T) was measured at a flow rate of 2 μl/min and a temperature of 25°C. After each run, immobilized ligand was regenerated by removing bound analyte with 5 μl of 0.1 M phosphoric acid at 20 μl/min, rinsing with 20 μl of PBS-T at 20 μl/min, followed by equilibration with PBS-T for 2 h at 2 μl/min. Both the association and dissociation kinetics were unaffected by variation of the flow speed (1–4 μl/min). Equilibrium dissociation constants were obtained by fitting a 1:1 association model to relative units (RU) values at t = 6000 s. Association and dissociation kinetics were fitted using the models provided with the Biacore analysis software.

Binding of IgG4 (natalizumab) was also measured. Binding was observed only after preincubation with glutathione (GSH) for 1 h at 37°C.

To investigate the effect of the regeneration cycle, IgG1 Fc and IgG4 Fc were immobilized (see above). After coupling, the chip was equilibrated for 24 h with PBS-T (2 μl/min). Binding of IgG4 (natalizumab preincubated with GSH) was measured two times at 2500 nM followed by a regeneration cycle; binding was measured two times after a regeneration cycle.

Size-exclusion chromatography

Samples (100 μl in PBS) were applied to a Superdex 200 HR 10/30 column (Amersham Biosciences), which was connected to a HPLC system (ÄKTAexplorer) from Amersham Biosciences. The column was first equilibrated in PBS followed by calibration with i.v. Ig (Sanquin) to determine the retention volumes of monomeric and dimeric IgG.

Results

IgG4 binds to solid-phase bound IgG1, but solid-phase bound IgG4 does not bind IgG1

Previously, IgG4 Fc binding was observed in assays with Fc or intact IgG coupled to a solid support (6) or to IgG subclasses in an immunoblot (9). In Fig. 2*A*, we compared binding of ^{125}I-labeled IgG1 or IgG4 to Sepharose-coupled IgG1 or IgG4. IgG4 binds both to coupled IgG1 and IgG4. Binding to IgG1 appears to be slightly more efficient, but IgG4 Fc binding during the coupling of IgG4 could reduce the capacity of the IgG4-Sepharose. Surprisingly, IgG1 does not bind to Sepharose-coupled IgG4, which implies the binding activity of IgG4 to be restricted to IgG coupled to a solid support. This suggests that IgG4 Fc-binding activity is directed either against epitopes that become available only after coupling or that binding requires multiple Fc fragments in close proximity (a high "packing density"). Similar results were obtained when IgG1 or IgG4 were coated onto polystyrene microtiter plates (Fig. 2*B*). Notably, to observe significant binding of IgG4, much more IgG has to be coated to Sepharose than to polystyrene (see *Discussion*). Several other monoclonal IgG1 Abs (infliximab, adalimumab, omalizumab) were coupled to Sepharose, all resulting in similar binding of IgG4 (supplemental supporting information[3]). The Ag specificity of the IgG4 Abs was also irrelevant (supplemental supporting information), as ^{125}I-labeled IgG4 Fc fragments bound IgG-coated Sepharose as efficient as intact IgG4 Abs (Fig. 3).

[3] The online version of this article contains supplemental material.

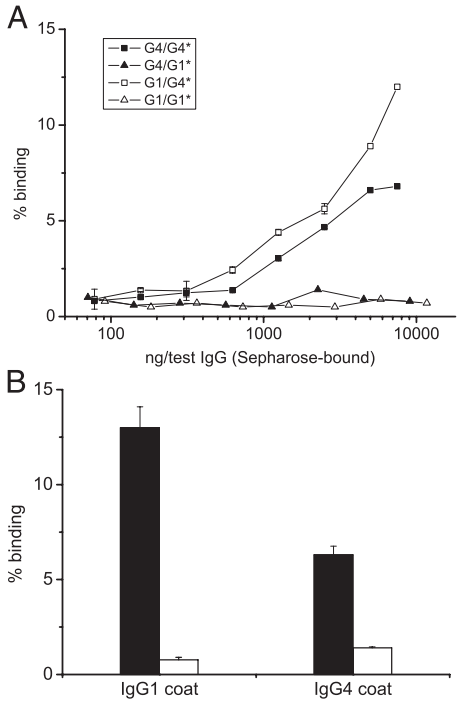

FIGURE 2. *A*, Binding of ^{125}I-labeled IgG1 or IgG4 (chimeric mAbs against Bet v 1) to Sepharose-bound IgG. Percentages are relative to total amount of labeled IgG added. Concentration of Sepharose was 0.6 mg/ml. IgG1 binding to IgG1 △, IgG1 binding to IgG4 (▲), IgG4 binding to IgG1 (□), and IgG4 binding to IgG4 (percent). *B*, Binding to IgG1(□) or IgG4 (■) immobilized onto microtiter plates.

Concentration, but not loading density of coupled IgG1 is important

Next, we investigated whether the Fc-binding activity of IgG4 depended on the IgG1 coupling density. In Fig. 3, binding of Fc fragments of IgG4 is plotted vs the amount of IgG1. Different coupling densities of IgG1 on Sepharose are compared. It is clear that the packing density is not a factor of importance. Rather, the absolute amount of coupled IgG is a factor that determines the amount of binding. Similar results were obtained using anti-Fel d 1 IgG4 instead of IgG4 Fc fragments (supplemental supporting information).

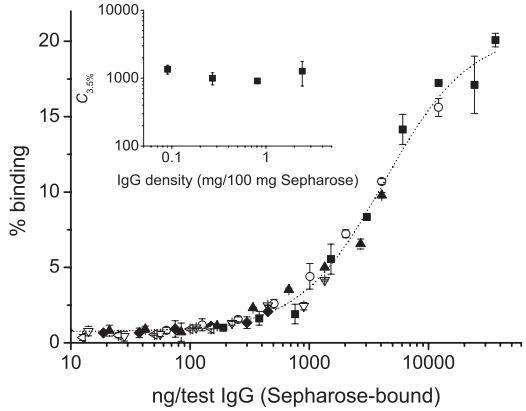

FIGURE 3. Binding of ^{125}I-labeled IgG4 Fc to IgG1 (adalimumab) coupled to Sepharose at different concentrations and loading densities. Densities of coupled Sepharose are (mg/100 mg Sepharose): 2.43 ■, 0.81 (○), 0.27 (▲), 0.09 ▼, 0.03 ◆, 0.01 ◁. *Inset*, Sigmoidal curves were fitted for different densities and the equivalent doses of IgG that result in 3.5% binding are plotted vs density.

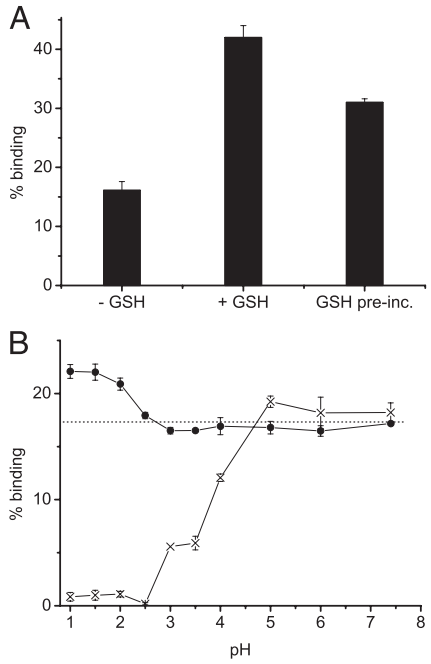

FIGURE 4. Conditions that influence IgG4 Fc binding to IgG1-Sepharose (*A*) in the absence or presence of 1 mM GSH, or after preincubation of IgG4 Fc with GSH (*B*) at different pH (×), or after preincubation and neutralization of IgG1-Sepharose at different pH (●).

Physicochemical factors that influence IgG4 Fc-Fc interactions

We tested different buffer conditions to learn more about the character of the IgG4-IgG1 Fc-Fc interactions. Variation of salt concentration between 0 and 2 M (NaCl) had no influence on the binding activity (data not shown). On the other hand, at a pH below 5, the binding was diminished and below pH 3, no binding was detected (Fig. 4).

Next, the influence of GSH, known to trigger the IgG4 half-molecule exchange, was investigated. It was found that in the presence of GSH, binding of IgG4 Fc to IgG1 increases >2.5-fold (Fig. 4). In the absence of GSH, but after preincubation of the radiolabeled IgG4 Fc with GSH, binding was increased 2-fold.

We explored the possibility that a conformational change of the solid phase-bound IgG1 such as partial dissociation of the CH3 domains leads to Fc-Fc interactions. A low pH-induced dissociation of isolated CH3 domains is described elsewhere (10). IgG1-Sepharose was preincubated for 30 min at a different pH between 1 and 7.4. Radiolabeled IgG4 Fc was added shortly (30–180 min)

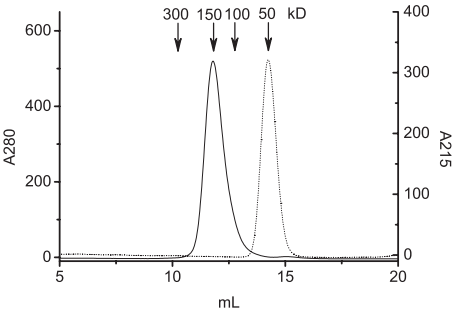

FIGURE 5. HP-SEC of IgG4 Fc at 8 μM (dashed line) and IgG4 at a concentration of 50 μM (solid line).

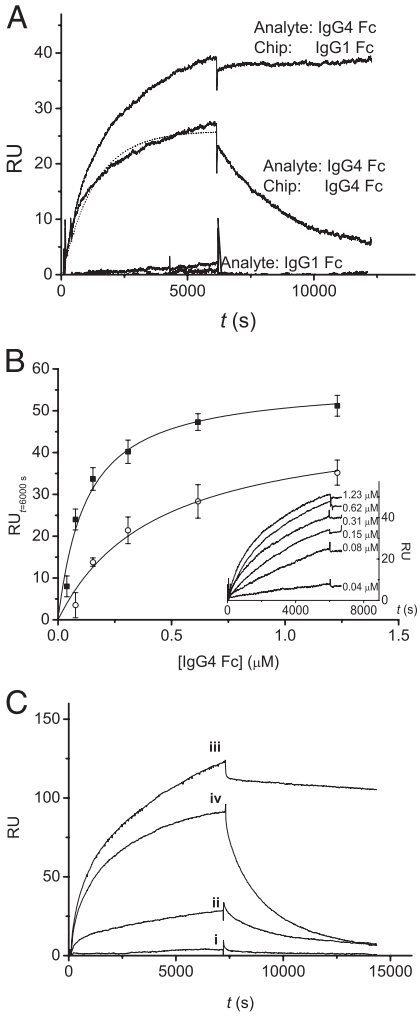

FIGURE 6. Biacore analysis of IgG4 Fc binding to IgG-Fc. *A*, IgG1 Fc and IgG4 Fc were injected at a concentration of 310 nM and a flow speed of 2 μl/min on a biosensor CM5 chip with IgG1 Fc or IgG4 Fc coupled (8000 and 4000 RU). $T = 25$°C. At $t = 6000$ s, analyte was replaced by buffer. IgG4 Fc binds reversibly to IgG4 Fc, but irreversibly to IgG1 Fc. *B*, IgG4 Fc was injected at concentrations between 40 and 1230 nM. End points ($t = 6000$ s) are plotted vs concentration of IgG4 Fc for IgG1 Fc (percent) and IgG4 Fc (○). A single site-binding model was fitted to the data. *Inset* shows corresponding sensorgrams for IgG1 Fc. *C*, IgG4 (2500 nM) was injected at 2 μl/min before any regeneration cycle with 0.1 M H_3PO_4 (*I*, IgG1 Fc; *ii*, IgG4 Fc) and after a regeneration cycle (*iii*, IgG1 Fc; *iv*, IgG4 Fc).

after neutralization to pH 7.4. This led to an increase of IgG4 Fc binding up to 30% following a pH shock below pH 2 (Fig. 4).

IgG4 Fc-Fc binding is not observed in solution

The fact that IgG4 binds to immobilized IgG1 or IgG4, whereas IgG1 does not bind to immobilized IgG4, suggests that immobilization plays a role in Fc-binding activity. However, IgG4 binding to IgG4 might not depend on conformational changes the same way as IgG4 binding to IgG1. Therefore, we studied Fc-Fc interactions in solution by size-exclusion chromatography (Fig. 5). At a concentration of 8 μM IgG4 Fc or 50 μM IgG4, <1% of dimers/oligomers was observed, with >99% of the Fc fragments or IgG molecules in monomeric form (50 and 150 kDa, respectively). Preincubation of IgG4 with GSH led to similar results. Assuming a simple 1:1 interaction, i.e., a monomer-dimer equilibrium, the K_d for dimerization was higher than 10 mM. This low-affinity interaction does not explain the IgG4 binding observed in the solid-phase assays.

Table I. *Apparent dissociation constants of IgG4 Fc to solid phase-bound IgG Fc, measured with SPR*

Solid Phase (/RU)	K_d^{eq} (μM)	k_{on} (s^{-1} M^{-1})	k_{off} (s^{-1})	K_d^{kin} (μM)
IgG1 Fc (8000)	0.12 (0.03)	459 (80)[a,b]	$<10^{-7}$	<1000
IgG4 Fc (4000)	0.43 (0.08)	309 (100)[b]	$3.3\ (1) \times 10^{-4}$	1.0 (0.3)

[a] Fitted with k_{off} set to 0.
[b] Average values; k_{on} is smaller at the highest concentrations of analyte (also apparent from initial slopes, *inset* Fig. 6).

IgG4 dissociates from IgG4, not IgG1, but association is similar

Fc-binding activity was analyzed by SPR. IgG1 or IgG4 Fc fragments were coupled to the biosensor chip and IgG4 Fc (natalizumab) or IgG1 Fc (adalimumab) were used as analyte. Slow association of IgG4 was observed; after 100 min, the association reaction was not yet complete (Fig. 6*A*). Compared with the amount of coupled Fc (4000–8000 RU), the amount of Fc binding IgG4 is modest (<1%). In other words, only a small fraction of the Fc fragments participates in binding. Apparent dissociation constants were estimated using a one-step association/dissociation binding mechanism to be 0.43 and 0.12 μM for IgG4 and IgG1, respectively, assuming 1:1 interaction (Table I and Fig. 6*B*). We found two striking differences between IgG4 binding to IgG1 and IgG4. First, with IgG4 Fc coupled to the chip, IgG4 Fc bound via Fc-Fc interactions dissociates slowly if flushed with buffer (Fig. 6*A*). A kinetic dissociation constant of 1.0 μM was calculated (Table I). In contrast, with IgG1 Fc coupled to the chip, no dissociation of IgG4 Fc was detected if flushed with buffer. However, complete dissociation was observed after regeneration with 0.1 M H_3PO_4 (data not shown). Second, we did not observe binding of IgG4 to IgG1 Fc directly after coupling, before any regeneration cycle with 0.1 M H_3PO_4 was conducted (Fig. 6*C*). By contrast, IgG4-IgG4 binding was observed also directly after coupling, albeit to a lesser extent (~35%). In other words, low pH treatment of the solid phase-bound IgG induces/enhances IgG4 Fc binding. It should be noted that binding remained fairly constant during repeated binding/regeneration cycles.

IgG4 Fc-Fc binding is inhibited by IgG4

Considering that the IgG4 Fc-binding activity often is an undesirable background reaction, it is of interest to further investigate possibilities to inhibit this type of reaction. It was found that binding of ^{125}I-labeled IgG4 to Sepharose-coupled IgG1 (Fig. 7) or IgG4 (data not shown) was inhibited by unlabeled IgG4. This demonstrates that IgG4 Fc binding to solid phase-bound IgG4 does not proceed indefinitely, i.e., IgG4 bound via Fc-Fc interactions was not available for further Fc-Fc interactions. Curiously, whereas the Fc fragment of natalizumab is a good inhibitor, natalizumab IgG4 inhibits significantly less efficiently compared with other IgG4 Abs. However, after preincubation with 0.5 mM GSH, all IgG4 Abs, including natalizumab, inhibited as efficiently as IgG4 anti-Bet v 1 (see *Discussion*).

Polyclonal human IgG was found to efficiently inhibit the IgG4 Fc-binding activity (Fig. 7*B*). It was also tested whether heat-aggregated (polyclonal) IgG was a better inhibitor than monomeric IgG. Classical RF binds poorly to monomeric IgG, but efficiently to polymeric IgG (11). By contrast, IgG4 Fc binding was inhibited equally well (less than a 2-fold difference) by monomeric and heat-aggregated IgG (Fig. 7).

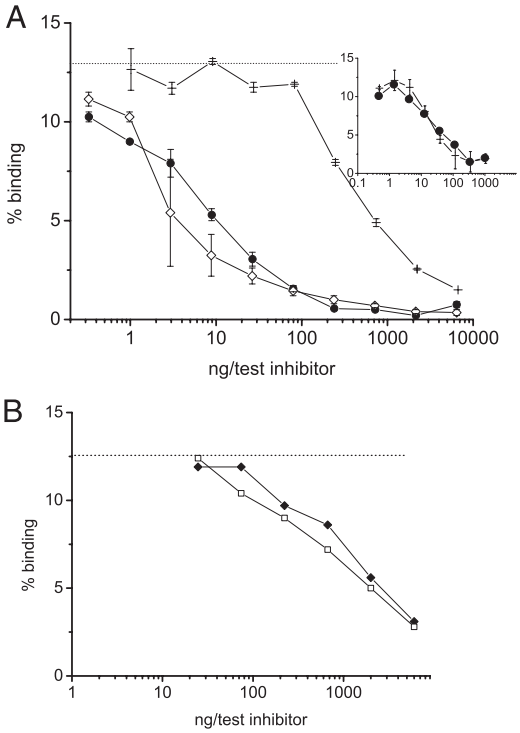

FIGURE 7. Inhibition of binding of ^{125}I-labeled IgG4 Fc to Sepharose-coupled IgG1. *A*, IgG4 anti-Bet v 1 (●), natalizumab (+), IgG4 Fc (natalizumab, ◇); *inset* shows inhibition after preincubation of inhibitor with 1 mM GSH. *B*, Polyclonal human IgG (i.m. IgG (◆) and heat-aggregated IgG (□). Dotted lines indicate percentage of binding without inhibitor.

In Ag-coupled assay, binding appears specific for IgG4

To test whether conformational changes in coupled IgG lead to new epitopes to which the IgG4 Fc-binding activity is directed, a "more physiological," indirect coupling to Sepharose was tested. Instead of coupling IgG directly (chemically) to Sepharose, it was captured via its Ag. Bet v 1-specific recombinant mAbs were captured by Bet v 1-coupled CNBr-activated Sepharose. Unlike the situation with IgG-coupled Sepharose (Fig. 1), where IgG4 bound to either IgG1- or IgG4-coated beads, we observed only binding of ^{125}I-labeled Fel d 1 IgG4 to Ag-captured Bet v 1-IgG4 (Fig. 8). No binding of ^{125}I-labeled Fel d 1 IgG4 was observed to Bet v 1-IgG1 in this assay.

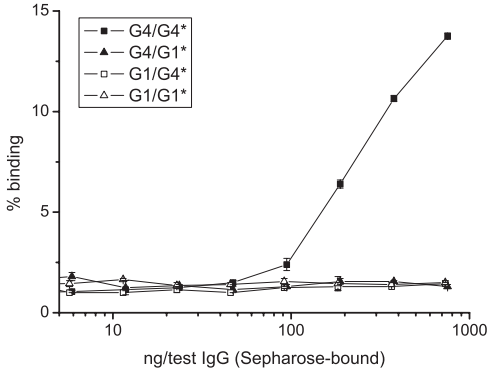

FIGURE 8. Binding of ^{125}I-labeled IgG4 or IgG1 (anti-Fel d 1) to IgG1 or IgG4 (anti-Bet v 1) bound to Ag-coupled Sepharose (Bet v 1). Per test, 0.6 mg of Sepharose was used (1.3 mg of Bet v 1/100 mg/Sepharose). IgG1 binding to IgG1 △, IgG1 binding to IgG4 (▲), IgG4 binding to IgG1 (□), IgG4 binding to IgG4 (percent).

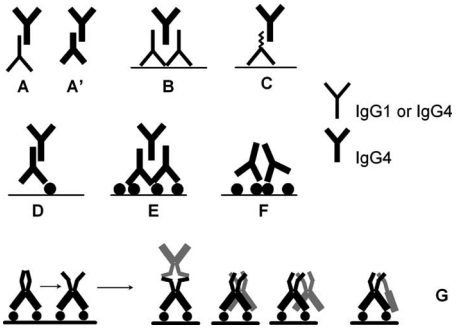

FIGURE 9. Models for the Fc-Fc interactions of IgG4. *A*, IgG4 binds to IgG Fc. *A′*, IgG4 binds to IgG4 Fc. *B*, IgG4 binds to multivalent IgG Fc. *C*, IgG4 binds to conformationally altered IgG Fc. *D*, Ag-bound IgG4 binds to IgG4 Fc. *E*, Ag-bound IgG4 binds to multivalent IgG4 Fc. *F*, IgG4 binding to surface Ag is reinforced by Fc-Fc interactions. *G*, Possible mechanism of interaction: CH3 domains dissociate; recombination with CH3 domains of another (IgG4) molecule results in an Fc-Fc interaction.

Discussion

The present results show that IgG4 Fc binding to either IgG1 or IgG4 is at least in part different phenomena. Furthermore, the binding appears to occur only if one of the binding partners is attached to a solid phase. Below we discuss 1) the role of the solid phase-bound IgG1 and IgG4, 2) which mechanism of binding can be deduced from the present data and does it bear relation to half-molecule exchange of IgG4?, and 3) the potential in vivo relevance.

Conformational changes in solid phase-bound IgG1 trigger IgG4 Fc binding

Which conditions lead to Fc-Fc interactions of IgG4 (Fig. 9)? The results of the HP-SEC indicate that IgG4 Fc-Fc interactions do not lead to dimer/oligomer formation (model *A′*, Fig. 9), and hence are weak or absent in fluid phase. Although IgG4 binds to IgG1 coated or chemically coupled to a solid support, IgG1 is not capable of binding IgG4 coupled to a solid support. Thus, the ability of IgG4 to bind to IgG is not inherent (Fig. 9, model *A* or *A′*) and requires specific conditions.

A plausible explanation for the solid phase-restricted IgG binding involves multiple Fc tails in close proximity that together result in submicromolar avidity as indicated in model *B* (Fig. 9).

However, for binding to IgG1, evidence does not support this hypothesis. Thus, heat-aggregated IgG should be a considerably more efficient inhibitor of the IgG4 Fc binding than monomeric IgG, which is not the case. Moreover, the density of IgG on the solid phase does not appear to affect the binding (Fig. 3). Therefore, this model is unlikely to explain binding to IgG1. For IgG4 coupled to Sepharose, it is difficult to quantify the binding because Fc-Fc interactions will form already during coupling.

A more reasonable explanation is that coupling of IgG to solid support results in partial unfolding of a fraction of the molecules (Fig. 9, model C). It is known that absorption by hydrophobic interactions leads to a large fraction (~90%) of structurally altered protein (12), i.e., possibly more IgG is unfolded such that IgG4 can bind. Indeed, immobilization of IgG to polystyrene resulted in a more efficient binding of IgG4 (Fig. 1). In a typical experiment, equal binding of labeled IgG4 is achieved with 3.75 μg of Sepharose-bound IgG1 (750 μl total volume) or 0.1 μg of polystyrene-bound IgG1 (150 μl total volume). Furthermore, incubating IgG1-Sepharose at a low pH ($\leq$ 2) results in a modest 30% increase in IgG4 Fc binding. Igs will undergo conformational changes while maintaining part of the secondary and tertiary structure in this pH

range (10, 13). In particular, dissociation of CH3 domains at this pH has been described (see below). Finally, IgG4 binds to IgG1 coupled to a biosensor chip only after a low pH treatment of IgG1. Therefore, we conclude that IgG4 binds only to a non-native conformation of IgG1.

IgG4 Fc-Fc interactions with solid phase-bound IgG4

Interestingly, binding to IgG4 coupled to a biosensor chip was also observed without a low pH treatment, albeit less. Therefore, IgG4 binding to IgG4 stands out from IgG4 binding to IgG1 in that an externally induced conformational change may not be required. At the same time, the enhanced binding by the low pH treatment points to a shared underlying mechanism of binding to both IgG1 and IgG4. Binding of IgG4 to Ag-bound IgG4 was also observed, providing further evidence that IgG4 binds to IgG4 whether its conformation is altered or not.

It is therefore puzzling that Fc interactions between IgG4 molecules are not observed in the fluid phase. Possibly, only IgG4 coupled at high densities results in Fc binding (Fig. 9, model *E*). However, experiments with IgG4 coupled at different densities to Ag-Sepharose did not support this hypothesis (results not shown). Below we present an alternative explanation: Fc interactions of IgG4 resemble an intermediate state of the half-molecule exchange reaction.

IgG4 Fc binding and half-molecule exchange

As stated in the Introduction, IgG4 is able to exchange half-molecules (Fab-arm exchange). This reaction proceeds in vitro under mildly reducing conditions (0.5–1 mM GSH). Encouraged by previous reports of IgG4 Fc-Fc interactions, we investigated whether association between IgG4 molecules may be considered a preequilibrium step in the exchange reaction. However, the present results do not unequivocally support this hypothesis. IgG4 binds with submicromolar affinity only to solid phase-bound IgG4. IgG4 remains essentially (>99%) monomeric in the fluid phase up to at least 50 μM, which translates to a dissociation constant >10 mM.

In contrast, our results suggest that Fc-Fc interactions may involve dissociation of an IgG4 molecule with subsequent recombination with a second, partially dissociated molecule of IgG4. In other words, Fc-Fc interactions may result from an incomplete exchange reaction, trapped because one molecule is bound to a solid phase. For this mechanism to be correct, CH3 domains of IgG4 (or IgG1) on the solid phase as well as of IgG4 in the fluid phase have to dissociate before binding is observed. On the solid phase, this condition can result, for example, from partial denaturation (see above). In the fluid phase, partial or complete dissociation appears to be restricted to IgG4 for two reasons that are discussed below: 1) IgG4 is in part a noncovalent dimer and 2) noncovalent forces between the C-terminal domains are weaker for IgG4 than IgG1.

Are IgG4 Fc-Fc interactions formed only by intrachain isomers of IgG4?

Up to 50% of IgG4 possesses intrachain disulfide bonds in the hinge, rather than interchain (1), as illustrated in Fig. 1. In the intrachain form, the H chains are held together only by noncovalent forces and are therefore in equilibrium with dissociated half-molecules. Theoretically, the intrachain form would be expected to exchange in the absence of GSH, but this was not observed (2). Probably, GSH catalyzes the exchange reaction for both the intra- and interchain form, which suggests that reduced cysteines play a role in the course of the exchange process other than simply being broken (possibly, CH1-CH2 interactions stabilize both the inter- and interchain form (1) but not the reduced form).

One striking result of the present study is the unusually low rate of association of IgG4 to chips coated with IgG. (A quantitative analysis of the kinetic data is beyond the scope of this article, but will be the subject of another article.) The slow association rate may result from a mechanism which involves a dissociation step of IgG4 into two half-molecules or a partial dissociation of the C-terminal domains. Interestingly, GSH enhances IgG4 Fc binding (Fig. 4*A*) and natalizumab is converted from a poor inhibitor to a good inhibitor with GSH (Fig. 7*A*). Natalizumab possesses a wild-type IgG4 hinge, as studied by Labrijn et al. (manuscript in preparation), and it contains few half-molecules (i.e., the intrachain form) on nonreduced SDS-PAGE (supplemental Fig. S4), but after preincubation with GSH, a significant proportion of intrachain IgG4 was observed. Taken these data together, it suggests that it is the intrachain form of IgG4 that binds to Fc.

Are IgG4 Fc-Fc interactions caused by CH3 dissociation?

A major contribution to the strength of the noncovalent intrachain dimer of IgG4 half-molecules will be the interactions between both CH3 domains. For IgG1, the K_d for dissociation of the CH3 dimer is $<10^{-10}$ M (14, 15). For IgG4, this interaction is most likely considerably weaker.

It can be speculated that weaker noncovalent interactions between the CH3 domains of IgG4 as compared with IgG1 allow partial dissociation of these domains and reassociation with CH3 domains of another IgG4 molecule (Fig. 9*G*). A mechanism involving extensive conformational alterations before binding would be in line with the slow association kinetics observed in the SPR experiments at 25°C. We hypothesize that the Fc-Fc interaction may be due to intercalation of CH3 domains from two Abs similar to the "domain swapping" observed of the V domains of a human IgG1 (16) and/or half-molecules that bind to partially dissociated solid phase-bound CH3 domains. These hypotheses are currently being investigated.

Possible biological implications

The present results suggest that binding of IgG4 to IgG1 is mainly an artifact induced by the assay format (i.e., coating IgG1 to a solid phase). On the other hand, Fc binding of IgG4 to IgG4 appears to be a more general phenomenon, perhaps closely related to the recently described Fab-arm exchange. At this point, one can merely speculate whether these interactions might be of relevance in vivo. Fc binding to Ag-bound IgG4 appears to be restricted to IgG4-IgG4 binding. It is possible that binding of IgG4 to Ag would selectively recruit additional IgG4 molecules to the site of action, e.g., to prevent inflammatory responses by shielding Fc tails. Alternatively, IgG4 Fc-Fc binding might serve as a mechanism to partly restore the avidity of IgG4 Abs, which in vivo are functionally monovalent due to half-molecule exchange, by binding cooperatively to Ag as well as to each other (Fig. 9, model *F*). The combination of monovalency and allosteric binding ensures a high avidity to (polyvalent) Ags, while at the same time reducing the potential of immune complex formation. Taking this argument one step further, the ubiquitous presence of GSH in vivo may cause Fc-Fc interactions of Ag-bound IgG4 to proceed to half-molecule exchange. The IgG4 molecules formed in this process will recognize different epitopes on the same protein and thus result in a much more stable 1:1 IgG4-Ag complex.

In summary, we demonstrated that IgG4 Fc-Fc interactions are limited to solid phase-bound IgG, i.e., IgG4 binds to solid-phase bound IgG1, but not vice versa. This limitation is most likely caused by the requirement of a conformational change in IgG1 before binding of IgG4. IgG4 binding to solid phase-bound IgG4

(chemically or via Ag capture) was found to depend less on externally induced conformational changes. We hypothesize that the Fc binding of IgG4 to IgG4 resembles the half-molecule exchange and may also occur in vivo to optimize the IgG4 response toward down-regulating inflammation.

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Disclosures

Authors specified here have a financial interest in Genmab: J.S. has stock and/or warrants and R.C.A. received consulting fees.

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