Dimers and multimers of monoclonal IgG1 exhibit higher in vitro binding affinities to Fcγ receptors

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Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; AUC, analytical ultracentrifugation; BSA, bovine serum albumin; CHO, chinese hamster ovary; HPLC, high performance liquid chromatography; HMW, high molecular weight; SPR, surface plasmon resonance; CD, circular dichroism; AUC, analytical ultracentrifugation; aFFF-MALS, asymmetric field flow fractionation—multi angle light scattering; DLS, dynamic light scattering

The in vitro binding of monomeric, dimeric and multimeric forms of monoclonal IgG1 molecules, designated mAb1 and mAb2, to the extracellular domains of Fcγ receptors RI, RIIA and RIIIB were investigated using a surface plasmon resonance (SPR) based biosensor technique. Stable noncovalent and covalent dimers of mAb1 and mAb2, respectively, were isolated from CHO cell expressed materials. The dissociation constants of monomeric mAb1 and mAb2 were determined to be 1 nM for the FcγRI-binding and 6–12 μM for the FcγRIIA- and FcγRIIIB-binding. Dimeric mAb1 and mAb2 exhibited increased affinities, by 2–3 fold for FcγRI and 200–800 fold for FcγRIIA and FcγRIIIB. Further increases in binding were observed when the antibodies formed large immune complexes with multivalent antigens, but not in a linear relation with size. The binding properties of monomeric mAb2 were identical with and without a bound monovalent antigen, indicating that antigen-binding alone does not induce measurable change in binding of antibodies to Fcγ receptors. Dimerization is sufficient to show enhancement in the receptor binding. Given the wide distribution of the low-affinity Fcγ receptors on immune effector cells, the increased affinities to aggregated IgG may lead to some biological consequences, depending on the subsequent signal transduction events. The SPR-based in vitro binding assay is useful in evaluating Fcγ receptor binding of various species in antibody-based biotherapeutics.

Introduction

Protein therapeutics, including monoclonal antibodies, have demonstrated increasing application in treating human diseases. The advantages of protein therapeutics, compared to traditional medicines composed of synthetic small molecules, include high specificity and low toxicity. However, due to their larger sizes and broad range of post-translational modifications, the risk of immunogenicity is elevated, especially when administered as multiple doses over prolonged periods.1,2 The generation of antibodies against protein therapeutics may cause reduction in their efficacy or alteration in clearance. More serious side effects would arise if the anti-therapeutic antibodies were to cross-react to endogenous proteins with essential biological functions.3–5

It has been shown that modifications in proteins, such as aggregation and chemical decomposition, may enhance the immune response.5,6 Synovial fluid from patients with rheumatoid arthritis contains both soluble and insoluble immunoglobulin aggregates which activate reactive oxidant production in human neutrophils.7 The interaction of soluble aggregates of IgA and IgG with rat mesangial cells triggered a number of responses, including release of inflammatory mediators, cell proliferation and catabolism of the complexes.8 Aggregated IgG and IgE, as well as their immune complexes with antigens, induced macrophage stimulation,9 and the efficiency of the macrophage stimulation correlated with the size of the IgG and IgE aggregates or their immune complexes.9,10 The activation of macrophages led to increased release of cytokines, lysosomal enzymes and nucleotides, as well as elevated antibody-dependent cell-mediated cytotoxicity (ADCC).11 Macrophage functions in inflammatory reactions and phagocytosis/endocytosis might be modulated as well. One possible consequence of the internalization of the aggregated or complexed Ig is the proteolytic breakdown of the Ig into peptides, which can be followed by binding of these peptides to class II major histocompatibility complex (MHC), activation of T cells and B cells by the peptide-MHC complexes, and the production of anti-Ig antibodies.12,13 The cause of the increased activation of immune cells by aggregated IgG, IgE or IgA was speculated to be the increased interactions with Fc receptors on those cells.10,14

The interactions between the Fc region of Ig molecules and Fc receptors (FcR) is one of the major signaling pathways in adaptive
activated by cytokines released from other activated effector cells. In contrast, FcγRIIA and FcγRIIIA and/or B are constitutively expressed on almost all leukocytes including lymphocytes B, T (subpopulation) and natural killer cells. FcγRIIA is capable of inducing most of the receptor-mediated effector cell activations by itself.24-27 The abilities of FcγRIII A and B to induce phagocytosis, ADCC and inflammation, have also been shown. Therefore, it is important to study the interactions with all three classes of Fcγ receptors when assessing the propensity of an IgG molecule to induce immune responses.

For an IgG antibody to mediate immune functions such as ADCC and phagocytosis, an antigen has to be recognized and captured by the complementarity-determining regions (CDRs) located invariably at the distal end of Fab domains from the Fc domain. A bridge is formed when an Fcγ receptor on the surface of the effector cells, which carry out ADCC or phagocytosis, binds to the Fc domain of the antigen-bound, or opsonized, IgG. To date, the exact location of the receptor binding site on Fc has been determined by x-ray crystallography for FcγRIII only.28,29 This receptor interacts simultaneously with both chains of Fc at the lower hinge and the NH₃-terminus of CH₂ domain near the glycosylation site. Studies using point mutations to characterize the binding properties of other receptors have suggested that there are common areas on the Fc surface for all three receptor classes.30

The enhanced binding of the aggregated IgGs or their large immune complexes to the Fc receptors expressed on the surface of various immune effector cells had been studied and reported.31-33 However, most of those cells express more than one class of the receptors. Even with blocking one or two classes using receptor-specific antibodies, it is difficult for those assays to address whether the binding to all types of receptors are enhanced, and, if yes, whether the enhancements are to similar extent. The recent availability of recombinant FcγRI, FcγRIIA and FcγRIIB extracellular domains has enabled us to study the binding affinity and kinetics of IgG molecules, as well as their variants, to individual receptors under identical conditions using in vitro methods. In this report, we show the enhanced binding of dimeric and oligomeric IgG1 forms, with and without multivalent antigens, to all three types of Fcγ receptor in a surface plasmon resonance (SPR)-based in vitro assay. The increase in the apparent activities correlated with the increase in the size of the aggregates or the immune complexes, consistent with the earlier observation using cell-based assays.11 In addition, we demonstrate that the effect is much greater for the low affinity receptors than for the high affinity receptor. The possible physiological implications and consequences of these differentiated effects are discussed.

Results

HMW characterization. The HMW fractions of mAb1 and mAb2 eluted from cHA column or SE-HPLC were heterogeneous in size. The size distributions were characterized using sedimentation velocity in analytical ultracentrifuge (AUC). Several species, from monomer to tetramer, were well-resolved when the data were analyzed with SEDFIT software (Fig. 1). In

Figure 1. Continuous distribution analysis of HMW mAb1 and mAb2. AUC sedimentation velocity data. The sedimentation profiles of the monomeric mAbs are shown as solid lines; the HMW fractions of each sample are dashed. HMW mAb1 (A) is predominantly dimer (80%), with minor amounts of monomer (6%), trimer (11%) and tetramer (3%). The HMW fraction of mAb2 (B) is also mainly dimer (70%) with a significant amount of monomer (23%) and approximately 7% higher molecular weight species. Data were analyzed using SEDFIT v. 8-9 software.37
indicate that alterations, if any, in the secondary structures upon either covalent or noncovalent dimerization was minimal. The spectra of both antibodies were consistent with a structure that is dominated by β-sheet, as expected for IgGs.

Antigen-binding of monomeric and HMW mAb1 and mAb2. The in vitro binding affinities of the HMW of mAb1 and mAb2 with their respective antigens, in comparison to their monomeric forms, were assessed using SPR. The HMW and monomeric mAb1 were individually captured at relatively low densities (~300 RU) by pre-immobilized protein A, and were allowed to interact with Ag1 at various concentrations. The dissociation phase was monitored for 5 minutes following each antigen injection, prior to the removal of all bound antibodies by a low pH solution. The “double-reference” corrected (see Methods), overlaid sensorgrams, depicting only the antigen-binding and dissociation phases, but not the capturing and the regeneration phases, are shown in Figure 4. Even though Ag1 was a bivalent analyte, and the mAb1 antibody, especially its HMW species, were bi-or multi-valent ligands, the binding kinetics could be adequately described by the Langmuir model (1:1 model) because the surface density and the analyte concentrations were kept sufficiently low. The resulting affinity constants (KD) and the R_max, which reflects the binding stoichiometry of the captured antibodies, are listed in Table 1. The HMW and the monomeric mAb1 exhibited very similar binding affinities for Ag1. The difference was within the experimental variability. However, a noticeable difference between the HMW and the monomeric mAb1 is the binding stoichiometry or capacity, reflected by the R_max values (Table

both mAb1 and mAb2, the main species was dimer, comprising 79 and 70%, respectively, of the total. The dimer of mAb1 was further isolated by SE-HPLC, and was used in the Fcγ receptor-binding tests. The HMW species of mAb2 had low abundance, as judged by SE-HPLC (data not shown) and SDS-PAGE (Fig. 2B, -DTT, sample “pool”). No further fractionation was performed for mAb2 HMW sample and the mixture was used in the in vitro binding tests.

The nature of the molecular association in the dimer and oligomers of mAb1 and mAb2 was investigated using SDS-PAGE. After heating in the presence of SDS, mAb1 HMW exhibited the same mobility as the monomer with and without a reducing reagent (Fig. 2A), indicating that the molecules in the HMW species were associated via non-covalent interactions. Despite the noncovalent nature, the species in HMW of mAb1 were very stable. After being stored at 4°C for one year, only a few percent of dimer converted to multimers, and the content of the monomer did not change. The majority of mAb2 HMW species exhibited lower mobility (higher apparent molecular weight) in the presence of SDS. A reducing reagent, such as DTT, was required to dissociate the HMW species (Fig. 2B), indicating that the molecules in mAb2 HMW species were covalently linked by disulfide bonds. The reduced SDS-PAGE also revealed minute amount of non-reducible HMW species, for which the structural characterizations are on going in our laboratory.

Far-UV CD spectroscopy was used to compare the secondary structures of mAb1 and mAb2 in the HMW fractions with that of monomers (Fig. 3A and B). The superimposable spectra indicate that alterations, if any, in the secondary structures upon either covalent or noncovalent dimerization was minimal. The spectra of both antibodies were consistent with a structure that is dominated by β-sheet, as expected for IgGs.

Antigen-binding of monomeric and HMW mAb1 and mAb2. The in vitro binding affinities of the HMW of mAb1 and mAb2 with their respective antigens, in comparison to their monomeric forms, were assessed using SPR. The HMW and monomeric mAb1 were individually captured at relatively low densities (~300 RU) by pre-immobilized protein A, and were allowed to interact with Ag1 at various concentrations. The dissociation phase was monitored for 5 minutes following each antigen injection, prior to the removal of all bound antibodies by a low pH solution. The “double-reference” corrected (see Methods), overlaid sensorgrams, depicting only the antigen-binding and dissociation phases, but not the capturing and the regeneration phases, are shown in Figure 4. Even though Ag1 was a bivalent analyte, and the mAb1 antibody, especially its HMW species, were bi-or multi-valent ligands, the binding kinetics could be adequately described by the Langmuir model (1:1 model) because the surface density and the analyte concentrations were kept sufficiently low. The resulting affinity constants (K_d) and the R_max, which reflects the binding stoichiometry of the captured antibodies, are listed in Table 1. The HMW and the monomeric mAb1 exhibited very similar binding affinities for Ag1. The difference was within the experimental variability. However, a noticeable difference between the HMW and the monomeric mAb1 is the binding stoichiometry or capacity, reflected by the R_max values (Table

Figure 2. Reduced and non-reduced SDS-PAGE of monomeric and HMW mAb1 (A) and mAb2 (B). Prior to loading, the non-reduced samples were heated to 70°C for 3 min in a buffer containing 2% SDS, 60 mM Tris-HCl, pH 6.8, 5 mM N-Ethylmaleimide. The reduced samples were heated to 100°C for 2 min in a buffer containing 2% SDS, 60 mM Tris-HCl, pH 9.0, 50 mM DTT. Both gels were run in a buffer containing 25 mM Tris, 192 mM glycine and 1% SDS.“Monomer” and “HMW”: SE-HPLC fractions that contained primarily monomeric and aggregated IgG. “Pool”: sample prior to SE-HPLC separation. The same molecular weight marker was used for gels in both (A and B).
**Figure 3.** Far-UV CD spectra for monomer and HMW mAb1 and mAb2. Spectra of monomeric (filled circles) and HMW (open squares) were recorded from 190–250 nm. For both mAb1 (A) and mAb2 (B), the HMW forms and the monomeric antibody have very similar far-UV CD spectra, suggesting that the native fold of the proteins was not significantly perturbed upon aggregation.
Figure 4 (See previous page). In vitro antigen-binding of mAb1 and mAb2 by SPR (Biacore). The monomer and HMW (see Fig. 1 for size distribution) samples of mAb1 (A and B, respectively) and mAb2 (C and D, respectively) were captured in CMS sensor chip by the pre-immobilized protein A at the amount of ~300 RU for mAb1 and ~1,000 RU for mAb2. The capturing efficiencies of the four samples were similar. The antigen solutions at varying concentrations, as indicated in the figures, were individually injected into the flow cells to allow binding with the captured antibodies. The protein A surface was regenerated following each binding, and the sensorgrams were corrected using a “double-reference” procedure (see Materials and Methods). The overlaid sensorgrams were globally fit to the Langmuir model (1:1 kinetics) to obtain the binding affinities. The thick grey curves are experimental data, and the thin black lines are fitted curves.

| antigen-binding affinities of monomeric and HMW mAb1 and mAb2 | mAb1 | mAb2 |
|---|---|---|
| | Monomer | HMW | Monomer | HMW |
| $k_+ \left( 10^5 \text{ M}^{-1} \text{s}^{-1} \right)$ | 4.70 | 3.84 | 4.45 | 4.37 |
| $k_- \left( 10^4 \text{ s}^{-1} \right)$ | 5.59 | 3.66 | 2.47 | 2.60 |
| $K_0 (\text{nM})$ | 0.074 | 0.065 | 0.045 | 0.045 |
| $R_{\text{max}}$ (RU) | 61.8 | 62.1 | 60.7 | 60.9 |
| Ave $K_0$ (nM) | 0.061 ± 0.008 | 0.11 ± 0.006 | 0.11 ± 0.006 | 0.11 ± 0.006 |
| Ave $R_{\text{max}}$ (RU) | 61.5 ± 0.4 | 34.4 ± 0.2 | 34.4 ± 0.2 | 34.4 ± 0.2 |

The affinity constants ($K_0$) and the fitted maximum signal ($R_{\text{max}}$), which reflects the binding stoichiometry, for mAb1 binding with Ag1 and mAb2 binding with Ag2 were determined in triplicate using SPR technology. Dimer or HMW species were captured in SPR flow cells in the same amount as the monomers, as determined by the increase in SPR response (RU) at the end of each capturing step. Global fitting of “double-reference” corrected sensorgrams to a 1:1 kinetic model (Langmuir) was employed (see Fig. 4). The average values and the Standard Error of the Mean for the fitted $R_{\text{max}}$ and the calculated affinity constants, from triplet measurements with separate reagent dilutions, are listed.

1). The fitted $R_{\text{max}}$ of HMW was close to 50% of that of the monomer. Since the captured HMW species contained the same number of IgG1 molecules as the captured monomers, judged by the SPR response at the end of each capturing step (all ~300 RU, equivalent to ~0.3 ng/mm²), the simplest conclusion would be that half of the binding sites in HMW species were totally inactivated, while the other half retained full binding affinity.

The 2.4 kDa Ag2 peptide was used in the mAb2 binding study. Since the mass of the peptide is low, the surface density was increased to improve the sensitivity. Approximately 1,000 RU of mAb2 or mAb2 HMW was captured by the protein A on a CM5 chip. Their respective sensorgrams, with curves for different antigen concentrations overlaid, are shown in Figure 4C and D. All curves fitted to a 1:1 kinetic model very well. The fitted $K_0$ and $R_{\text{max}}$ for the monomeric and the HMW mAb2, listed in Table 1, are not significantly different.

Size distributions in the immune complexes of mAb1 and mAb2. Because intact IgG are bivalent for antigen-binding, if the antigens are not excessively large, then bi- or multi-valent antigens can concatenate multiple IgG molecules to form large chain- or ring-shaped immune complex (see Fig. 6A). Ag1 is naturally a covalent homodimer. The Ag1 stock solution was titrated into mAb1 stock to result in a solution of 70 nM mAb1 and 70 nM Ag1 at a neutral pH. The size distribution in the mixture was analyzed using asymmetric field flow fractionation (aFFF), in line with UV absorption for concentration and multi-angle light scattering for size determination. Although complexes of different sizes were not completely resolvable by aFFF, the continuous increase in the apparent size of the mixture (Fig. 5, open circle) and the decrease in the mAb1 monomer peak area (Fig. 5, first peak of the solid curve vs. dashed curve) indicate the formation of large complexes involving mAb1, with the majority comprising 2–3 units of mAb1 + Ag1 (Fig. 5, second peak of the solid line, mass of 4–6 x 10⁵ Da) and the largest on the order of ~60 units (mass of 10⁷ Da).

To make the Ag2 peptide a multi-valent antigen, the peptides were chemically conjugated to BSA with multiple attachments per BSA molecule. A series of solutions with varying ratios of Ag2-BSA to mAb2 were made to further demonstrate the formation of concatimers and verify the concatenation scheme for a double-bivalent system as illustrated in Figure 6A. However, the size distribution in the immune complex of mAb2 with Ag2-BSA could not be analyzed with aFFF due to the non-specific Ag2 binding to the aFFF membrane. Batch mode dynamic light scattering in a 96-well plate format was used to obtain the weight averaged particle size in each solution, Figure 6B. As the ratio of Ag2-BSA to mAb2 increased from 0 to 1, the increase in the turbidity could be observed visually, as shown in the top panel of the figure. The opposite trend was seen as the ratio increased from 1 to 2, consistent with the concatenation scheme. However, the
to kinetic models to resolve association and dissociation rate constants is bound to have much higher uncertainty. However, the establishment of a steady-state (plateau in each sensorgram) due to the fast dissociation provided an alternative approach to the affinity measurement. The steady-state responses at varied analyte concentrations were obtained by averaging the SPR signals over the selected period of time (horizontal bars) in the overlaid, “double-reference” corrected, sensorgrams (Fig. 8A), using BiaEvaluation tools. The corresponding binding isotherm (averaged steady-state responses vs. analyte concentrations, Fig. 8B) was used to fit the following binding equilibrium for $K_D$, also using the manufacturer provided software:

$$A + B \leftrightarrow AB$$

for which the dissociation constant is given by

$$K_D = \frac{(R_{max} - R_{eq})C}{R_{eq}} \text{ (M)}$$

where $R_{eq}$ is the steady-state response corresponding to the analyte concentration $C$, and $R_{max}$ is the fitted maximum response. This approach requires a much wider range of analyte concentration to reach saturation of immobilized ligand.

The steady-state measurement was performed for the binding of monomeric mAb1 and mAb2 with both FcγRIIA and FcγRIIIB. Figure 8 shows the binding of mAb1 to FcγRIIIB as an example. The fitted $K_D$ are listed in Table 2. Again, the mAb1 and mAb2 monomers had very similar binding affinities for FcγRIIA, as well as for FcγRIIIB, as expected for the identical Fc sequence. The affinities for FcγRIIIB were slightly lower than that for FcγRIIA.

Upon dimerization (100 and 70% dimer in the samples of mAb1 and mAb2, respectively), the off-rates of mAb1 and mAb2 binding to FcγRIIA and FcγRIIIB were all significantly lower, indicating increased binding affinities. As a result, a steady-state was no longer reached within normal injection time, and the kinetic approach to data acquisition and analysis was required.

Figure 9 shows mAb2 HMW binding with FcγRIIA as an example. Due to the heterogeneity in the molecular weight and the potential multivalent binding, the sensorgrams could not fit the simple 1:1 binding model satisfactorily. The “two-state” model, also provided in BiaEvaluation, was used to obtain the apparent binding affinities for mAb1 and mAb2 dimers binding to FcγRIIA and FcγRIIIB:

$$A + B \leftrightarrow AB'$$

where $K_{A1}$ is the dissociation constant, $k_a$ and $k_d$ are the fitted association and dissociation rate constant, respectively.

$$K_A = \frac{k_a}{k_d} \text{ (M)}$$

$$K_{A1} = k_1/k_d$$

$$K_{A2} = k_2/k_d$$

$$\text{Apparent } K_A = K_{A1}(1 + K_{A2}) \text{ (1/M)}$$

The affinities of IgG1 for FcγRIIA and FcγRIIIB are known to be much weaker than for FcγRII, with $K_A$ in the micromolar range. The dissociation rate constants were so high that the binding of mAb1 and mAb2 monomers to receptors on the sensor exhibited a fast-on and fast-off kinetic profile. Fitting this type of sensorgrams

DLS measurements revealed that significant amount of multimers remained even beyond the 2:1 ratio.

Binding of monomer and dimer/HMW of mAb1 and mAb2 with Fcγ receptors RI, RIIA and RIIIB. To facilitate the analysis of Fcγ receptor-binding, the dimer of mAb1 was further isolated from the HMW mix. This was not done for mAb2 due to the limited quantity of the HMW material.

The binding of mAb1 dimer and mAb2 HMW, compared with their monomers, to FcγRI was measured using the kinetic method of SPR. All the overlaid sensorgrams (Fig. 7) were globally fit with Langmuir model (the 1:1 model):

$$A + B \leftrightarrow AB \quad K_{dij} = k_d/k_a \text{ (M)}$$

where $K_{dij}$ is the dissociation constant, $k_a$ and $k_d$ are the fitted association and dissociation rate constant, respectively.

The SPR signals of the dimer of mAb1 and the HMW of mAb2 were normalized based on monomer molecular mass (treating HMW of mAb2 as 100% dimer). The fitted dissociation constants are listed in Table 2. Consistent with the fact that the Fc regions of mAb1 and mAb2 are identical in sequence, the dissociation constants of the two monomers binding with FcγRI were nearly identical. Upon dimerization (100 and 70% dimer in the samples of mAb1 and mAb2, respectively), the dissociation rates were visibly decreased, and the fitted affinities of the FcγRI-binding increased by a factor of 3–5.

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Apparent $K_{a} = 1/$Apparent $K_{a} (M)$

The results are listed in Table 2. Compared to monomers, the apparent affinities of mAb1 and mAb2 dimers for FcγRIIA and FcγRIIIB increased by 2–3 orders of magnitude.

The Fcγ receptor-binding of the immune complex of monomeric mAb2 with monomeric peptide antigen Ag2 were also studied. All the sensograms could overlay with the corresponding sensograms in the absence of Ag2 and fit well to the 1:1 kinetic model. The fitted kinetic parameters and the binding constants were identical to that of monomeric mAb2 in the absence of Ag2, indicating that the antigen-binding in Fab domain did not affect the receptor-binding at the hinge region.

**Binding of large immune complexes of mAb1 and mAb2 with Fcγ receptors RIIA and RIIIB.** To investigate whether the apparent affinity to Fcγ receptors increases further when more antibody molecules are associated, immune complexes made of mAb1 with bivalent Ag1, and mAb2 with multivalent Ag2-BSA conjugate that were larger than dimer or HMW species of self-associated antibodies were tested using the same SPR method. The size distributions in the immune complexes were examined using static or dynamic light scattering, shown in Figures 5 and 6B. Figure 10 shows the sensograms of FcγRIIA- and FcγRIIIB-binding to these large complexes, overlaid with that to the dimer and monomer of mAb1 and mAb2. The total concentration of IgG (250 nM) in the dimers and in the immune complexes was calculated using the corresponding monomer molecular weight. Therefore, all samples contained the same amount of IgG binding sites for the receptors. Equimolar antigens were present in each immune complex in this experiment. The SPR signal of the dimers was normalized based on the monomer molecular weight, treating the HMW of mAb2 as 100% dimer. However, the normalization was not performed for the immune complexes due to the high degree of the heterogeneity. It is apparent that the dissociation rates of the immune complexes of both IgG1s, binding with both FcγRIIA and FcγRIIIB, decreased further compared with that of the dimers, thus indicating a further increase in the apparent affinities.

A trend in the weight averaged size was observed in the immune complexes of mAb2 with Ag2-BSA conjugates when the ratio of the antigen:antibody increased from 0:1 to 2:1 (see Fig. 6B). The averaged size seemed to be the largest when the ratio approached to 1, then decreased to a smaller value consistent with trimer or tetramer. A reasonable scenario for the phenomenon is the formation of concatemers, illustrated in Figure 6A. The FcγRIIA- and FcγRIIIB-binding of this series of complexes were tested using the SPR method. The results for
were already on the order of $10^{-9}$–$10^{-10}$ M (Table 2), approaching the instrument limit. Further increases in the affinities, possibly induced by the increase in the number of associated antibodies, would unlikely be detected in the sensorgrams.

Discussion

An in vitro method using SPR technology was employed to study the binding of individual Fcγ receptors, RI, RIIA and RIIIB, to various molecular forms of IgG1, which included monomers, covalent and noncovalent dimers and oligomers, as well as concatemers bridged by multivalent antigens (i.e., immune complexes). The approach was designed to (1) test the feasibility of using biosensor technology to detect differences among the Fcγ receptors, and in the molecular forms of IgG molecules; (2) determine the binding constants for homogeneous materials; (3) demonstrate that the increase in Fcγ receptor-binding upon

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**Figure 7.** In vitro binding affinities of mAb1 and mAb2 for FcγRI by SPR (Biacore). FcγRI with a His-tag at the C-terminus was captured in a CMS sensor chip, at ~400 RU, by the pre-immobilized anti-His antibody. Solutions of the monomer and the dimer of mAb1 (A and B, respectively), monomer and HMW of mAb2 (C and D, respectively) at varying concentrations, as indicated in the figures, were individually injected into the flow cells to allow binding to the captured FcγRI. The anti-His surface was regenerated following each binding, and the sensorgrams were corrected using a "double-reference" procedure (see Materials and Methods). The overlaid sensorgrams were globally fit to the Langmuir model (1:1 kinetics) to obtain the binding affinities. The thick grey curves are experimental data, and the thin black lines are fitted curves.
Table 2. In vitro Fcγ receptor-binding affinities of monomeric and HMW mAb1 and mAb2

| Sample  | Monomer   | Dimer   | Monomer   | HMW (70% dimer) |
|---------|-----------|---------|-----------|-----------------|
|         | mAb1      | mAb2    |           |                 |
| FcγRII  | kᵣ (M⁻¹s⁻¹) | 7.1E5   | 6.7E5     | 6.0E5           | 3.6E5           |
|         | kₐ (s⁻¹)  | 6.4E-4  | 6.1E-4    | 6.2E-4          | 7.3E-5          |
|         | kᵣ (M⁻¹)  | 9.1E-10 | 9.2E-10   | 1.0E-10         | 1.1E-9          |
| Ave kᵣ (M⁻¹) | 9.2E-10 | 1.6E-10 | 1.0E-9    | 2.9E-10         |
| FcγRIIB | kᵣ (M⁻¹s⁻¹) | SS      | SS        | SS              | SS              |
|         | kₐ (s⁻¹)  | SS      | SS        | SS              | SS              |
|         | kᵣ (M⁻¹)  | 5.9E-6  | 6.1E-6    | 8.0E-9          | 5.9E-6          |
| Ave kᵣ (M⁻¹) | 6.0E-6   | 7.9E-9  | 6.0E-6    | 2.3E-8          |
| FcγRIIB | kᵣ (M⁻¹s⁻¹) | SS      | SS        | SS              | SS              |
|         | kₐ (s⁻¹)  | SS      | SS        | SS              | SS              |
|         | kᵣ (M⁻¹)  | 1.1E5   | 1.1E-5    | 5.6E-8          | 8.5E-6          |
| Ave kᵣ (M⁻¹) | 1.1E-5   | 5.4E-8  | 8.8E-6    | 5.4E-8          |

The affinity constants (Kᵣ) for mAb1 and mAb2 binding with three classes of Fcγ receptors were determined in triplicate using SPR technology. Global fitting of “double-reference” corrected sensorgrams to a 1:1 kinetic model (Langmuir) was used for all samples binding to the high-affinity receptor FcγRI. A steady-state method, involving fitting the isotherms to a 1:1 equilibrium equation, was used for the binding of monomeric mAb1 and mAb2 to low-affinity receptors FcγRIIA and FcγRIIB. Global fitting to a “two-state” model (see Results) was used for binding to the low-affinity receptors FcγRIIA and FcγRIIB of mAb1 dimer and mAb2 HMW. Duplicate measurements for each sample are shown. *Less accurate due to heterogeneity in molecular weight. SS: Steady-state measurements.

Figure 8. Steady-state analysis of the in vitro binding of monomeric mAb1 to FcγRIIB. FcγRIIB with a His-tag at the C-terminus was captured in a CMS sensor chip, at ~400 RU, by the pre-immobilized anti-His antibody. Solutions of monomeric mAb1 at various concentrations, as indicated in (A), were individually injected into the flow cells to allow binding to the captured FcγRIIB. The anti-His surface was regenerated following each binding, and the sensorgrams were corrected using a “double-reference” procedure (see Materials and Methods). The sensorgrams were overlaid (A), and the steady-state values for each concentration, averaged over a chosen region as indicated by the horizontal lines, were plotted against the concentration to obtain the binding isotherm (B). The equilibrium binding constant was obtained by fitting the binding isotherm to the equilibrium binding equation (see text) using BiaEvaluation. The solid line in (B) is the fitted curve.
multimerization of IgG, observed with immune cells, takes place on the biosensor with immobilized recombinant receptors; (4) address whether the size dependence of IgG-Fc receptor-binding is the same for the three classes of receptors.

**System characterization.** Previous estimates of binding affinities of IgG for Fcγ receptors ranged from micromolar to nanomolar, well within the optimal working range of Biacore instruments. Our results for two monomeric monoclonal IgG1 molecules, shown in Table 2, are wholly consistent with those estimates. The strategy of uniformly capturing a molecule on the biosensor with immobilized recombinant receptors; (4) multimerization of IgG, observed with immune cells, takes place on the biosensor with immobilized recombinant receptors; (4) address whether the size dependence of IgG-Fcγ receptor-binding is the same for the three classes of receptors.

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Large immune complexes of mAb1 with bivalent Ag1 and of mAb2 with multivalent Ag2-BSA conjugate were prepared and studied in this work as a model for large aggregates. There is evidence that soluble aggregates, generated by heat or other means, bear many similarities to this type of immune complex including capability to activate immune cells, and clearance via the reticuloendothelial system. The size distribution of the immune complex of mAb2 exhibited a dependence on the antigen:antibody ratio. The averaged size increased, but peaked at ~1:1, and then declined, as the ratio increased. This is expected of such complexes as illustrated in Figure 6A. The concentrations of the immune complex used in the SPR assay had to be much lower than that in light scattering due to difference in instrument sensitivities. Based on the measured affinity for mAb2-Ag2 binding, $K_D = 10 \text{nM}$ (Table 1), the concentration used in the receptor-binding measurements, 250 nM, was sufficient to retain the size distributions observed by light scattering at higher concentrations. Additional relevance of our in vitro study of large immune complexes can be exemplified by antibody therapeutics against transforming growth factor superfamily members, which are often dimeric. In this case, large immune complexes formed in circulation, depending on the binding affinity and relative abundance of the antibody and antigen, may behave differently from single antibody-monovalent antigen complexes.

**In vitro binding affinities.** Using the capturing-binding procedure and the above described materials, we were able to carry out comparative studies for the interactions of higher molecular weight species of mAb1 and mAb2 with the three Fcγ receptors. We first compared the dissociation constants for the binding of monomeric and dimeric antibodies to FcγRI, FcγRIIA and FcγRIIB. The purified dimer of mAb1 and the preparation of mAb2 containing ~70% dimer yielded similar results. Compared with the monomers, binding to all three receptors was enhanced, consistent with cell-based results, which showed that dimer bound more than monomer, and trimer bound more than dimer, to immune cell surfaces. However, since the cells used in these studies might express all three receptors on the surface, it is not clear if the enhancement in binding was through one or more receptors, and if the contribution from each receptor class was equal in these cell-based assays. Robinson, et al. used antibodies to specifically block FcγRII or FcγRIIIB in their study of the activation of human neutrophils by natural aggregates from patients with rheumatoid arthritis, and concluded that the activation occurred via cooperative occupancy of both FcγRII and FcγRIIIB. However, the questions regarding the interaction of aggregates with FcγRI remained open since FcγRI has very low or no expression on human neutrophils before the neutrophils are activated. Our method allowed quantitative comparisons among individual receptors, showing for the first time, and with two types of dimer, that the enhancement in dimeric mAb binding to FcγRI is less profound compared to FcγRIIA and FcγRIIIB. The apparent affinities to FcγRIIA and FcγRIIIB increase by several orders of magnitude, from ~10 µM, which is not detectable by cell-based assays, to ~10–20 nM, comparable to that of mAb binding to its peptide antigen (Table 1).

It is evident in Figure 10 that the dissociation rates were further decreased when large immune complexes of mAb1 and mAb2, formed in the presence of multivalent antigens, were brought into contact with FcγRIIA and FcγRIIIB. Since the small immune complex of single mAb2 with monomeric Ag2 did not show...
a limit to the size of the complex in which all antibody molecules could actively participate in binding to the cell receptors; with larger complexes an increasing proportion of antibody molecules would become redundant. We tested this postulation by varying the antibody/antigen ratio in the mAb2/Ag2-BSA complex, which had varying averaged sizes, as shown by dynamic light scattering. With constant receptor density on the sensor and antibody concentration in the solution, we observed a similar phenomenon. The increase and decrease in binding signals paralleled the increase and decrease in weight averaged size as the antigen:antibody ratio increased from 0:1 to 2:1. However, the dissociation rates were approximately the same for all the ratios (Fig. 11). This means that, as the average number of mAb2 per complex increased, the observed changes in binding signal reflected mainly the changes in the averaged mass bound per binding event (similar to the effect of molecular weight change), but not in the avidity-based affinity.

Type III hypersensitivity is an immune complex-induced immune reaction, the manifestations of which include a

any increase in affinity compared to mAb2 alone, the observed enhancement with large complex must be due to the increase in size, rather than the presence of antigens. This is supported by a recent study with linearly connected Fc domains, which showed by ELISA assay augmented binding affinities to FcγRIIA, FcγRIIB and FcγRIIIA in the absence of the antigen. The change in the FcγRI-binding was to much lesser extent. Although the sensorgrams shown in Figure 10 could fit the “conformational change” model provided in Biacore software reasonably well, we did not report the fitting results since a binding constant would be ill defined in this case due to the heterogeneity in size of the complexes. Nonetheless, the significant decrease in the rate of dissociation evidenced by the overlaid sensorgrams is sufficient to provide basis for qualitative assessments.

The trend of increasing affinity with an increase in size was previously observed from monomer to dimer and to trimer, up to seven IgG molecules. With larger constructs, the apparent affinity to macrophages exhibited a plateau or even declined slightly. The researchers of those studies inferred that there was a limit to the size of the complex in which all antibody molecules could actively participate in binding to the cell receptors; with larger complexes an increasing proportion of antibody molecules would become redundant. We tested this postulation by varying the antibody/antigen ratio in the mAb2/Ag2-BSA complex, which had varying averaged sizes, as shown by dynamic light scattering. With constant receptor density on the sensor and antibody concentration in the solution, we observed a similar phenomenon. The increase and decrease in binding signals paralleled the increase and decrease in weight averaged size as the antigen:antibody ratio increased from 0:1 to 2:1. However, the dissociation rates were approximately the same for all the ratios (Fig. 11). This means that, as the average number of mAb2 per complex increased, the observed changes in binding signal reflected mainly the changes in the averaged mass bound per binding event (similar to the effect of molecular weight change), but not in the avidity-based affinity.

Type III hypersensitivity is an immune complex-induced immune reaction, the manifestations of which include a
combination of symptoms, including fever, weakness, rashes, arthritis and lymphadenopathy. It was believed that the development of such reactions involves the interactions of antibody complexes with molecules of the complement system, which activate effector cells such as mast cells and neutrophils. Recent findings that Fcγ receptors expressed on these cells are important or critical to some autoimmune diseases and inflammation suggest an alternative or parallel pathway for the role of protein aggregates and large immune complexes in immune cell activation.15,48 The elevation in the apparent affinity to FcγRIIA and FcγRIIIB, by as much as 3–4 orders of magnitude observed in this work, may provide support, and even a mechanism.

It should be noted that by either pathway, the activation of immune cells by large immune complexes might affect therapeutic antibodies against endogenous circulating targets that are naturally dimeric or multimeric, such as many cytokines in tumor necrosis factor (TNF) and transforming growth factor (TGF) superfamilies. Some measures to reduce the Fcγ receptor and complement binding potency of these molecules, such as mutating key amino acid residues in the binding sites,18,30 may be desirable.

To summarize, the SPR-based biosensor assay was used to analyze the high-affinity (nanomolar range $K_D$) and low-affinity (micromolar range $K_D$) binding interactions between immunoglobulins and their Fcγ receptors. The technology is sensitive enough to detect the effects of conformation/composition of IgG molecules or complexes. Our results showed enhanced binding of dimers and oligomers of IgG to Fcγ receptors compared to monomers, which corresponds to previously reported results of in vivo or cell-based assays. The observed size-dependence is more significant for the low-affinity FcγRIIA and FcγRIIIB compared to the high-affinity FcγRI. Similar enhancement was not found when mAb2 was bound to its monovalent antigen. However, binding with a multivalent antigen enhanced the receptor binding of both mAb1 and mAb2 due to the formation of multi-unit complexes. The consequences of the enhanced binding could include enhanced Fcγ receptor-induced immune reactions, such as ADCC, phagocytosis and inflammation. The size-dependence could be modulated by the composition and conformation of the IgG aggregates/complexes. The in vitro assay used in this work provides a simple method to evaluate the Fcγ receptor binding for therapeutic antibodies and Fc-fusion proteins.

**Materials and Methods**

**Materials.** Wyeth proprietary monoclonal antibodies, mAb1 (fully human IgG1 with $\lambda$ light chains, against a 25 kDa protein Ag1) and mAb2 (humanized IgG1 with $\kappa$ light chains, against a 24-amino acid polypeptide Ag2), were expressed in Chinese Hamster Ovary (CHO) cells. The antibodies, secreted into the media, were purified first with a protein A column, followed by an ion exchange chromatography, ceramic hydroxyapatite (cHA) for mAb1 or trimethyl aminoethyl (TMAE) for mAb2. The high molecular weight (HMW) species of mAb1 had stronger interaction with the cHA column than the monomers and had to be eluted with 1 M salt. Size exclusion HPLC (SE-HPLC) was employed to enrich the HMW species from the purified mAb2, which was in low abundance.

The antigen of mAb1, Ag1, is a transforming growth factor (TGF) superfamily member, and naturally a homodimer covalently linked by inter-chain disulfide bonds. The recombinant Ag1 was expressed in CHO cells and purified using a Ni-NTA Superflow nickel chelate column, followed by SE-HPLC in the presence of 6 M urea at pH 2.5, then the reversed phase
HPLC. The purified Ag1 was stored in 0.1% trifluoroacetic acid (TFA).

The antigen of mAb2, Ag2, is a chemically synthesized 24-amino acid peptide. The multivalent antigen, Ag2-BSA, was generated through reaction of iminothiolane treated BSA (yielding sulfhydryl containing BSA) with mercaptopropionic acid (MPA) treated Ag2 peptide. The end product was heterogenous, with variable numbers of antigen coupled to each BSA molecule.

The recombinant extracellular domains of human FcγRI (Gln16—Pro288,34), FcγRIIA (Gln34—Ile218,35) and FcγRIIIB (Gly17—Gln208,36), expressed in the mouse myeloma cell line NS0 and with His-tags at their C-termini, were purchased from R&D Systems. Since the various isoforms of both FcγRIIA and FcγRIIIB have high sequence homology in their extracellular domains, and they differ significantly only in the intracellular and the transmembrane domains, the isoforms will not be distinguished in this report. The anti-His antibody was purchased from Qiagen.

**Analytical ultracentrifugation (AUC) sedimentation velocity.** Samples, 0.5 mg/mL in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂PO₄, 1.5 mM KH₂PO₄, pH 7.2), were equilibrated from 20°C and centrifuged at 38,000 RPM for 10 hrs in a Beckman XL-A analytical ultracentrifuge. The rate of protein sedimentation was monitored using absorbance at 280 nm.

The sedimentation data were analyzed using Sedfit (v. 8-9)37 to determine sedimentation coefficients and approximate molecular masses. Sedimentation coefficients were corrected to reflect standard conditions, pure water at 20°C.38

**Circular dichroism (CD) absorption spectroscopy.** Far-UV CD spectra of samples, 0.1 mg/mL in 10 mM phosphate, pH 7.2, were acquired using an Aviv Model 202 spectrometer. Data were collected from 250–190 nm using a 0.1-cm quartz cell, equilibrated at 20°C.

**aFF-MALS and dynamic light scattering.** The size distribution of mAb1-Ag1 complex was analyzed using Wyatt Technologies Eclipse 2 asymmetrical-flow field flow fractionation system (aFFF) for protein separation, and tri-detector system, consisting in series with a Waters 2487 UV absorbance detector, a Wyatt Technologies DAWN multi-angle light scattering detector (MALS), and a Wyatt Technologies Optilab rEX differential refractometer, for protein analyses. The aFFF system incorporated a 490 μm spacer and a regenerated cellulose membrane with a molecular mass cut-off of ~30 kDa. The samples were injected at a flow rate of 0.2 mL/min subjected to a focusing flow of 2.0 mL/min. The separation began with a flow rate of 1.0 mL/min and a cross-flow of 2.0 mL/min. The cross-flow was linearly decreased to 0 mL/min in 20 minutes. The data were analyzed using Wyatt Technologies Astra (v5.1.9) software.

The size distribution of mAb2-Ag2 complexes were analyzed using the Wyatt Technologies DynaPro dynamic light scattering (DLS) instrument (formerly Protein Solutions). The instrument is equipped with a 96-well plate reader. Samples were prepared with increasing molar ratios of antigen to antibody, from 0:1 to 2.5:1, at 0.25 increments. The mAb2 concentration was kept constant at 5.0 mg/mL.

**Surface plasmon resonance.** Biacore 3000 and CM5 chips were used in the study of mAb1 and mAb2 binding to Ag1 and Ag2. Z-domain protein A (GE Healthcare) was pre-immobilized in two flow cells (-1,500 RU) using the amine-coupling kit from Biacore, Inc. One antibody or antibody derivative was injected into one of the flow cells to be captured by the protein A. The amount of capturing, also referred to as the surface density, was controlled by the concentration of the antibody solution, the flow rate and the injection time. Immediately following the capturing of an antibody, the appropriate antigen, at a chosen concentration, was injected into both flow cells. The flow cell without an antibody was used as the surface control, and the injection of buffer was used as the injection control. Each binding sensorgram from the sample flow cell, containing a captured antibody, was corrected for both the surface blank and the buffer injection controls (double-reference).39 Following each injection of an antigen solution, which induced the binding reaction, and the dissociation period, during which the running buffer was infused, the protein A surface was regenerated by the injection of a solution containing 10 mM Na₂PO₄, pH 2.5 and 500 mM NaCl. All captured antibodies, with and without antigens bound, were completely removed, and another cycle could begin. More than 200 cycles could be performed without apparent loss in the capturing capacity of the immobilized protein A.

CM5 chips and a similar procedure were also used in the study of mAb2 and mAb binding to FcγRI, FcγRIIA and FcγRIIIB (R&D Systems). The procedure is the same as the above described for the antigen binding, except that the capturing reagent was an anti-His antibody (Penta-His, Qiagen), immobilized in both the sample and the control flow cells (-4,000 RU); the His-tag containing Fcγ receptors were captured by the immobilized antibody and the antibodies or antibody derivatives under investigation were last injected as the analyte. The double-reference treatment and the regeneration conditions were also the same as the above.

All measurements were performed at 25°C, with a flow rate of 30 μL/min for kinetic measurements and 10 μL/min for steady-state measurements. The running buffer for all measurements was HBS-P (Biacore, Inc.) containing 0.01 M HEPES, pH 7.4, 0.15 M NaCl, 0.005% polysorbate 20. All data analyses were performed using the instrument manufacturer supplied software BiaEvaluation. Various kinetic models and fitting methods in the software were applied to different experiments based on the binding characteristics; these are described in the Results section.

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