Affinity and Kinetic Analysis of Fcγ Receptor IIIa (CD16a) Binding to IgG Ligands*

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Binding of pathogen-bound immunoglobulin G (IgG) to cell surface Fcγ receptors (FcγRs) triggers a wide variety of effector functions. The binding kinetics and affinities of IgG-FcγR interactions are hence important parameters for understanding FcγR-mediated immune functions. We have measured the kinetic rates and equilibrium dissociation constants of IgG binding to a soluble FcγRIIIa fused with Ig Fc (sCD16a) using the surface plasmon resonance technique. sCD16a interacted with monomeric human IgG and its subtypes IgG1 and IgG3 as well as rabbit IgG with on-rates of $6.5 \times 10^3, 8.2 \times 10^4, 1.1 \times 10^4$, and $1.8 \times 10^4$ M$^{-1}$ s$^{-1}$, off-rates of $4.7 \times 10^{-3}, 5.7 \times 10^{-3}, 5.9 \times 10^{-3}$, and $1.9 \times 10^{-2}$ s$^{-1}$, and equilibrium dissociation constants of 0.72, 0.71, 0.56, and 1.1 μM, respectively. The kinetics and affinities measured by surface plasmon resonance agreed with those obtained from real time flow cytometry and competition inhibition binding experiments using cell surface CD16a. These data add to our understanding of IgG-FcγR interactions.

Fcγ receptors (FcγRs)$^7$ are a family of cell surface glycoproteins with varying affinities for the Fc region of immunoglobulins G (IgG). Three classes of human FcγRs have been described: FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16), which are widely distributed in hematopoietic cell lineages. These include at least 12 different isoforms many of which are polymorphic. For example, CD16 has two isoforms, a and b, that differ by six amino acids in the extracellular domain and the presence (CD16a) or absence (CD16b) of the transmembrane and cytoplasmic domains (1).

The IgG-FcγR interaction-mediated binding of antibody-opsonized pathogens to leukocytes is a key event by which antibody effector functions are initiated. Kinetic rates and binding affinity are critical determinants of an IgG-FcγR interaction, as they control how likely and how rapidly such binding will occur, how many bonds will be formed, and how long the bonds last. In addition, it has been hypothesized that these parameters are related to the signaling events following the initial binding (2–4). Using a micropipette method, we have measured the kinetic rates and binding affinities of several IgG-FcγR interactions when both interacting molecules are anchored on apposing surfaces. We found that in this assay the half-lives of these IgG-FcγR bonds are in the order of seconds (5–9).

In addition to interaction parameters of surface-linked molecules, the kinetic rates and affinities of FcγRs for soluble IgG are also of interest, because in vivo interactions of immobilized IgG to leukocyte FcγRs are subject to competitive binding of soluble antibodies in sera. Of the three FcγRs, CD64 binds to monomeric IgG with high affinity ($K_d \sim$ tens of μM) (10), CD32 and CD16b are of low affinities ($K_d$ several and tens of μM, respectively) (6, 11, 12), and CD16a is considered as an intermediate affinity receptor ($K_d$ hundreds of μM) for monomeric IgG (6, 10, 12). In addition to the above results, which were obtained by conventional assays using FcγR-expressing cells, there were two studies using the surface plasmon resonance (SPR) technology and soluble FcγRs. Galon et al. (13) reported half-lives of the order of 10 min and equilibrium dissociation constants of several micromolar for sCD16b$^{NA2}$ binding to human (h) IgG1 and IgG3. By comparison, Maenaka et al. (14) found a much faster off-rate (half-lives of the order of seconds) but similar $K_d$ for IgG-SCD16b$^{NA2}$ binding.

Here, we report kinetic and affinity measurements of a soluble dimeric human FcγRIIIa (sCD16a) interacting with various IgG ligands in both monomeric and multimeric forms using SPR. We found that sCD16a interacted with monomeric rabbit (Rb) IgG, hIgG, hIgG1, and hIgG3 with on-rates of $1.8 \times 10^8$, $6.5 \times 10^8$, $8.2 \times 10^3$, and $1.1 \times 10^4$ M$^{-1}$ s$^{-1}$, off-rates of $1.9 \times 10^{-2}, 4.7 \times 10^{-3}, 5.7 \times 10^{-3}$, and $5.9 \times 10^{-3}$ s$^{-1}$, and equilibrium dissociation constants of 1.1, 0.72, 0.71, and 0.56 μM, respectively. sCD16a bound with aggregated ligands with lower apparent equilibrium dissociation constants and slower apparent off-rates. To ensure that the relatively slow kinetics is not artifactual, various controls were performed in both the experiment and analysis steps. The slow kinetics was not found to be artifacts because of the mass transport limitation, ligand aggregation, or dimeric binding of the sCD16a molecule. The kinetic
rates and affinities were also measured by real-time flow cytometry and a competition inhibition binding experiment using CD16 expressed on the cell surface, which were found to be in agreement with those obtained by SPR.

**EXPERIMENTAL PROCEDURES**

**Cells, Soluble CD16a, and Antibodies—Chinese hamster ovary (CHO) cells transfected to express human CD16α<sub>TM</sub>, CD16β<sub>NA1</sub>, CD16β<sub>NA2</sub>, and B7-1<sup>SPI</sup> as well as untransfected CHO cells were cultured as previously described (15, 16). sCD16 was generated by attaching the extracellular domain of CD16α to the Fc domain of IgG1. The mutated IgG1 CH2-CH3 Fc domains were obtained from Dr. Peter Linsley, Bristol-Meyer Squibb, as the hB7-1-lg construct and the extracellular domain of hCD16α was cloned in the place of hB7-1 as described (17). Mutations in the Fc domain of hCD16α-lg are L267F, L268E, G270A, and A363T (numbered as in accession number AAH69020.1). These mutations were shown to abolish the binding of FcyRs (18, 19). The anti-CD16 nonblocking monoclonal antibody (mAb) 214.1 (murine IgG1) was a generous gift from Dr. Howard Fleit (State University of New York, Long Island). The anti-CD16 adhesion blockade mAb CLBFcgran-1 (murine IgG2a) was purified in house from hybridomas as previously described (20). Cleavage of CLBFcgran-1 into Fab fragments was done by Lampire (Pipersville, PA). The rabbit anti-mouse Fc polyclonal antibody was purchased from BIAcore (Piscataway, NJ). Total hIgG and subtypes (hIgG1, hIgG2, and hIgG3) as well as RbIgG were purchased from Sigma, except hIgG1 used for the real time flow cytometry experiment, which was a generous gift from Dr. Adrian Whitty (Biogen Inc., Boston, MA). Fab of CLBFcgran-1 and hlgG1 was labeled with fluorescein isothiocyanate (Molecular Probes, Eugene, OR) following the manufacturer’s instructions.

**Size Exclusion Chromatography—**Monomeric and multimeric IgG ligands were separated by size exclusion chromatography. 7.5 g of Sephadex G-200 (Amershams Biosciences) was swelled in 200 ml of PBS/EDTA (containing 5 mM EDTA, pH 7.4) at 90 °C for 5 h and then cooled at 4 °C overnight. The supernatant was decanted and the gel was resuspended in 150 ml of PBS/EDTA and poured into a column. Two columns were used, with respective diameters of 1.7 and 1.0 cm and respective volumes of 150 and 103 ml (Bio-Rad). The columns were rinsed with 300 ml of PBS/EDTA at 0.3 ml/min between each run. Two sets of gel filtration standards were used to calibrate the columns. The first set included 4 mg each in 1 ml of PBS/EDTA of cytochrome c (molecular mass 12 kDa), blue dextran (2,000 kDa), and BSA (88 kDa) (Sigma). The second set included 2.5 mg of thyroglobulin (670 kDa), 2.5 mg of bovine γ-globulin (158 kDa), 2.5 mg of chicken ovalbumin (44 kDa), 1.25 mg of equine myoglobin (17 kDa), and 0.25 mg of vitamin B12 (1.4 kDa). After adding 5–20 mg of IgG in 1 ml of PBS/EDTA, the column was connected to a 1-liter reservoir of PBS/EDTA. Setting the flow rate at 0.25–0.75 ml/min, the effluent was collected sequentially in fractions of 1.5 ml each. The optical density at 280 nm was measured to monitor the protein concentration in each fraction.

Monomeric or multimeric IgG fractions were used immediately after separation, either directly or further diluted to lower concentrations for SPR or real time flow cytometry experiments. In some cases where higher concentrations were desired, chromatographed IgG was reconstituted by using protein concentrators (Amicon, Beverly, MA). The concentrations of monomeric and multimeric IgG were determined by a protein estimation kit (Bio-Rad).

**SPR Measurement—**SPR experiments were conducted in a BIAcore<sup>TM</sup> 1000 instrument using CM5 sensor chips (BIAcore) at 25 °C. Samples were prepared in PBS/EDTA and perfused over the sensor chip at 30 μl/min (unless otherwise stated) for kinetic measurements. For other control experiments, the flow rate was 5 μl/min. The running buffer was BIA-certified HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% Surfactant p20) filtered and degassed using 0.2-μm bottle top filter (Millipore, Bedford, MA).

Several approaches were tried in preliminary experiments to immobilize sCD16a on the sensor chip, including the standard amine coupling procedure to coat either sCD16a directly or a nonblocking anti-CD16 mAb 214.1, which in turn captured sCD16a. Unfortunately, the former adversely affected the ability of sCD16a to bind ligands and the latter did not allow satisfactory regeneration of the sensor chip. The approach that worked best with our reagents appeared to be coupling first a rabbit anti-mouse Fc antibody to the sensor chip with the amine coupling procedure then binding mAb 214.1, followed by capturing sCD16a. This approach also ensured the consistent orientation of the sCD16a molecule on the sensor chip. The sensor chip could be regenerated with three washes of 10 μl of 1 M formic acid (Sigma), which completely removed noncovalently bound sCD16a and 214.1 without impairing the reactivity of the anti-mouse Fc antibody. The binding of mAb 214.1 was ≥95% of the initial level after 40 times of regeneration. Using the full rabbit anti-mouse Fc antibody yielded negligible sCD16a binding to its Fc region, as confirmed by a control experiment in which sCD16a was injected over the sensor chip coupled with the full rabbit anti-mouse Fc antibody alone without mAb 214.1 followed by subsequent injection of IgG ligands.

The kinetic rates of IgG-sCD16a binding were derived by globally fitting the Langmuir (1:1) model (cf. Equations 1 and 2) to the family of association and dissociation curves collected at different ligand concentrations, using BLAevaluation 3.0 software provided by the manufacturer. The affinity of CLBFcgran-1 Fab-sCD16a binding was derived by Scatchard analysis.

**Kinetic Measurement of Cell Surface CD16 by Real Time Flow Cytometry—**5 × 10<sup>6</sup> cells were washed three times in 5 ml of binding buffer (RPMI 1% IgG-free BSA, 0.02% sodium azide). Cells were resuspended in 0.5 ml of binding buffer and transferred to a tube. Fluorescein isothiocyanate-labeled ligands in binding buffer were added and quickly mixed with the cells to obtain 2.5 × 10<sup>5</sup> cells/ml cell concentration and the desired final ligand concentration. A range of ligand concentrations was selected based on separate affinity measurements using other techniques, such that the receptors could be saturated at high concentrations yet the ligands would not be depleted in low concentrations. To assay for association, the sample was immediately run in a FACS Vantage machine (BD Biosciences) and a total of 15 fluorescence intensity histograms were measured sequentially in predetermined time points over a 12-min
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period. The earliest time point a histogram could be measured was ~10 s. Each histogram included >2,000 events and took ~2 s to acquire. The sample was vortexed between two consecutive time points to ensure cells and ligands were well mixed. The right-shift of the histogram toward higher fluorescence intensity was monitored in real time.

To assay for dissociation, the remaining sample from the association assay was incubated for another 20 min to ensure equilibrium was achieved. 1 ml of the sample was centrifuged, the supernatant was decanted, and the tube was vortexed to disperse the cell pellet. 2 ml of plain medium was added to resuspend the cells. The sample was immediately run in the FACS machine to measure another 15 fluorescence intensity histograms sequentially in predetermined time points over a 12-min period.

The background-subtracted mean fluorescence intensity (MFI) versus time data of the association and dissociation assays were, respectively, fit to equations:

\[ C = \frac{k_{on} LR_T}{k_{on} + k_{off}} \{1 - \exp[-(k_{on}L + k_{off})t]\} \quad \text{(Eq. 1)} \]

and

\[ C = \frac{\eta k_{on} LR_T}{k_{on} + k_{off}} \exp(-k_{off}t) \quad \text{(Eq. 2)} \]

where \( k_{on} \) and \( k_{off} \) are the respective on- and off-rates and \( t \) is time. The concentration of bound ligands \( C \) is proportional to the measured MFI. Neglecting depletion due to binding, the free ligand concentration \( L \) is assumed to be the same as that added to the reaction mixture. \( R_T \) is the concentration of total receptors. The proportionality constant \( \eta \) accounts for the difference between the equilibrium MFI from the association assay and the initial MFI from the dissociation assay.

The standard free energy of binding, \( \Delta G^\circ \) (kcal mol\(^{-1}\)), was calculated from the \( K_a \) measured at various temperatures, \( \Delta G^\circ = RT \ln(K_a) \), where \( R \) is the universal gas constant (1.987 \times 10^{-3} \text{ kcal mol}^{-1} \text{ K}^{-1} \), \( T \) is the absolute temperature, and \( K_a \) is expressed as mole liter\(^{-1}\).

**Affinity Measurement for Cell Surface CD16**—The affinities of CLBFcgran-1 Fab for CHO cell CD16\(^{TM} \), CD16\(^{hNA1} \), and CD16\(^{hNA2} \) were determined by Scatchard analysis. The affinities of monomeric hlgG to CD16 were measured by a competitive inhibition binding assay as previously described (6). Briefly, CHO cells were grown in flasks until near confluence. Cells were rinsed once in PBS and then removed from the flask using PBS/EDTA. After washing, they were resuspended at 10\(^6\) cells/ml in PBS/EDTA and added to V-bottom 96-well plates at 100 \( \mu \text{l/well} \). The wells were precoated with 1% IgG-free BSA (Sigma) in PBS by incubating at room temperature for 2 h. They were rinsed with PBS/EDTA and kept on ice until the cells were added. After adding cells, the plates were spun at 2000 rpm for 2 min. The supernatant was removed and 50 \( \mu \text{l} \) of IgG in PBS/EDTA at the titrated concentrations were added to each well with mixing. Then, 50 \( \mu \text{l} \) of \(^{125}\text{I}-\text{CLBFcgran-1 Fab in PBS/EDTA at a concentration of 0.25-0.50 } \mu \text{g/ml} \) was added to each well, followed by a 45-min incubation on a shaker at 5 °C. After washing 3 times, the cell pellets were removed and counted in a \( \gamma \) counter.

In the presence of increasing concentrations of the low affinity ligand (IgG, concentration \( c_l \)), the binding of the high affinity ligand (\(^{125}\text{I}-\text{CLBFcgran-1 Fab, concentration } c_f \)) to the cell surface receptor (CD16) is gradually reduced, or displaced. The displaced fraction, defined as the bound fraction \( /f \) of CLBFcgran-1 normalized by the value when no IgG was present \( (f_0) \), can be expressed by Equation 3.

\[ \frac{f}{f_0} = \left[ c + c_l + \left( c c_f + 1 / K_a \right) / 2 c \right] - \left( \left[ c + c_l + \left( c c_f + 1 / K_a \right) / 2 c \right] - 4c c_l \right)^{1/2} / 2 c \quad \text{(Eq. 3)} \]

Because the affinity to CD16 of \(^{125}\text{I}-\text{CLBFcgran-1 Fab (} K_a \) and the receptor concentration \( c \) were predetermined from a separate experiment by Scatchard analysis, the only unknown in Equation 3 is the affinity of IgG \( (K'_a) \). Therefore, \( K'_a \) can be calculated from a single measurement of \( f \) without the experimental displacement curve to include data at the IC\(_{50}\) point. To increase the accuracy of the \( K'_a \) value, however, the predicted displaced fraction (Equation 3) was nonlinearly fit to the entire \( f/f_0 \) versus \( c_l \) data set.

**RESULTS**

**Demonstration of Binding Specificity**—As shown in Fig. 1, monomeric hlgG did not bind to a CM5 chip surface only coated with rabbit anti-mouse Fc antibody and mAb 214.1. However, after further functionalization with sCD16a, a similar injection of monomeric hlgG resulted in a time-dependent binding of 370 resonance units in 150 s. Switching the perfusate from hlgG solution to plain buffer resulted in dissociation of the bound hlgG. The chip was regenerated by washing off the 214.1-sCD16a-HlgG complex with three injections of 1 M for-
mic acid at 2 min each, and then re-capturing mAb 214.1 and sCD16a. Binding resulted when the saturating concentration of CLBFeGRan-1 Fab (anti-CD16 blocking mAb) was injected; however, subsequent injection of monomeric hlgG did not yield further binding (not shown), indicating nearly complete blockade of the sCD16a sites for hlgG binding. These results indicate that the binding of hlgG to the surface coated with sCD16a was mediated by the specific hlgG-sCD16a interaction.

Separation of Monomeric and Multimeric IgG—The presence of multimers in commercial IgG even after high speed centrifugation was revealed by size exclusion chromatography as exemplified in Fig. 2A for RbIgG. Results for hlgG, hlgG1, and hlgG3 were similar (data not shown). The first peak, which appeared at fraction 28, was eluted at nearly the same time as the peak fraction of blue dextran (indicated by arrow). Blue dextran has a molecular mass (2,000 kDa) much higher than that of monomeric IgG (∼150 kDa) and was excluded by Sephadex G-200. This suggests that the first peak represents multimeric RbIgG. A second peak of RbIgG, which appeared at fraction 40, was eluted before BSA (peaked at fraction 51). The relative elution volumes (Ve/Vo) of cytochrome c, BSA, and IgG (2nd peak, assumed to be monomeric) are plotted against their molecular weights in Fig. 2B and compared with published results (21). Good agreement is seen between our data and those of Andrews (21), indicating that the second IgG peak in Fig. 2A indeed represents monomers. The small difference in the relative volume of cytochrome c between the results of Andrews (21) and ours may be attributed to the difference in how long the column had been used, which was noted to influence the relative position of low molecular weight protein. As shown in Fig. 2A, the multimeric RbIgG comprised a significant fraction of RbIgG from the commercial source, which would have adversely affected the SPR measurement if not removed.

To determine whether the chromatographed IgG would form aggregates in experiments where reconstituted IgG was used, the monomeric RbIgG fractions were pooled, reconstituted to 5 mg/ml, and passed through the column again in the time scale of SPR experiments. It is evident from Fig. 2A that only a single, symmetric peak of monomeric IgG was seen, apparently free of any detectable multimers. This suggests that re-aggregation of IgG is a relatively slow process and did not occur under our experimental conditions, which is consistent with a previous report (12).

Kinetics of Monomeric IgG-sCD16a Interactions—Monomeric IgG solutions of increasing concentrations were perfused over the sCD16a-derivitized sensor chip and the interaction time courses were measured, as exemplified in Fig. 3. It can be seen that both the association and dissociation were relatively slow, requiring a few minutes to achieve equilibrium or reach half-dissociation. It is also apparent that RbIgG bound to and dissociated from sCD16a faster than human IgGs.

sCD16a was immobilized to the sensor chip via a nonblocking anti-CD16 mAb 214.1, which in turn was captured by a rabbit anti-mouse Fc antibody. Although at rates much slower than those of IgG ligands dissociating from sCD16a, sCD6a and 214.1 also dissociated from their respective capturing antibodies over time, manifested as a negatively drifting baseline. To correct for this effect, the curve resulting from an injection of plain buffer alone, in which the slow dissociation of 214.1 and sCD16a was monitored, was subtracted from all the measured binding curves. The refraction index difference between protein samples and the running buffer, manifested as an instantaneous resonance unit change, was also determined in a control experiment (on the same rabbit anti-mouse Fc antibody-coated chip but without sCD16a immobilization) and subtracted from raw data in all sensorgrams presented here.

A Langmuir (1:1) model was fit simultaneously to the entire family of data curves in both association and dissociation
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FIGURE 3. Binding of monomeric IgG to scCD16a. Monomeric total hlgG (A), RbIgG (B), hlgG3 (C), and hlgG1 (D) at respective concentrations of 2.56, 3.29, 2.99, and 2.00 μM (top curves at the corresponding panels) as well as four serial 2× dilutions (progressively lower curves at the corresponding panels) were perfused over immobilized scCD16a at a flow rate of 30 μl/min for 150 s, followed by an injection of plain buffer. Both association and dissociation phases are shown in the overlaid sensorgrams after subtraction of the baseline drift and the refraction index difference between protein samples and the running buffer. RU, resonance units.

TABLE 1
Kinetic rates and equilibrium dissociation constants of scCD16a for IgG measured by SPR

| Ligand          | k<sub>on</sub> 10<sup>-9</sup> M<sup>-1</sup> s<sup>-1</sup> | k<sub>off</sub> 10<sup>-3</sup> s<sup>-1</sup> | K<sub>d</sub> μM |
|-----------------|-----------------|-----------------|-------------|
| Monomeric       |                 |                 |             |
| hlgG (n = 6)    | 6.51 ± 0.26     | 4.71 ± 0.24     | 0.72 ± 0.02 |
| hlgG1 (n = 4)   | 8.18 ± 0.28     | 5.74 ± 0.36     | 0.71 ± 0.04 |
| hlgG3 (n = 4)   | 10.64 ± 1.34    | 5.89 ± 0.33     | 0.56 ± 0.09 |
| RbIgG (n = 5)   | 17.62 ± 3.75    | 18.6 ± 2.52     | 1.07 ± 0.16 |
| Multimeric      |                 |                 |             |
| hlgG (n = 3)    | 12.67 ± 0.93    | 1.98 ± 0.14     | 0.16 ± 0.02 |
| hlgG1 (n = 3)   | 15.59 ± 1.13    | 1.19 ± 0.02     | 0.077 ± 0.006 |
| hlgG3 (n = 3)   | 13.19 ± 0.24    | 0.67 ± 0.02     | 0.051 ± 0.001 |
| RbIgG (n = 3)   | 9.27 ± 0.15     | 1.03 ± 0.02     | 0.11 ± 0.002 |

phases. Such a global fitting procedure accentuates deviation at any one concentration but ensures more robust kinetic constants overall (22). The kinetic rates so evaluated are summarized in Table 1; and the model predictions based on these parameters are exemplified in Fig. 4 along with the data. It can be seen that the model fits both the association and dissociation data of the hlgG-scCD16a interaction reasonably well. Similarly good fits were obtained for the hlgG1- and RbIgG-scCD16a interactions (data not shown). By comparison, the fit for the hlgG3-scCD16a interaction is not as good (data not shown). Despite its low concentration in serum (23), hlgG3 is known to comprise at least 12 allotypes (24). In this study, no attempt was made to further separate the various hlgG3 allotypes from the purchased heterogeneous mixture, which, we believe, is the reason why the homogeneous Langmuir model does not fit the hlgG3-scCD16a interaction data well.

Kinetics of Multimeric IgG-scCD16a Interactions—Compared with monomers, multimeric IgG dissociated from scCD16a much more slowly and required much lower concentrations to achieve the same level of binding, indicating a much higher avidity (Fig. 5). For the purpose of comparing our results with data in the literature, the same Langmuir model was used to fit the association and dissociation curves of IgG multimers. The kinetic mechanism for binding of multimeric ligands must not be 1:1; as such, a more involved model is needed to extract the intrinsic kinetic constants. Fitting the simple Langmuir model

FIGURE 4. Global fitting of association and dissociation curves of hlgG-scCD16a interaction. A Langmuir (1:1) model was globally fit to the hlgG sensogram in Fig. 3A measured at 2.56 μM (top, and top curve) as well as four serial 2× dilutions (progressive lower curves at the corresponding panels) to minimize the rebinding effect, only the first 60 s in the dissociation data (right panel) were used. The beginning and the end of the association data (left panel) were also excluded from the curve fitting as recommended by BIAevaluation 3.0. The experimental data are shown as points in every 5 s and the model fits are plotted as shown curves. The k<sub>on</sub> and k<sub>off</sub> values so estimated as well as the number of repeated experiments (n) for each ligand are listed in Table 1. RU, resonance units.

FIGURE 5. Binding of multimeric ligands to scCD16a. Multimeric total hlgG (A), RbIgG (B), hlgG3 (C), and hlgG1 (D) at respective concentrations of 1.53, 2.33, 1.66, and 0.97 μM (top curves at the corresponding panels) as well as four serial 2× dilutions (progressive lower curves at the corresponding panels) were perfused over immobilized scCD16a at a flow rate of 30 μl/min for 150 s, followed by an injection of plain buffer. Both association and dissociation phases are shown in the overlaid sensorgrams after subtraction of the baseline drift and the refraction index difference between protein samples and the running buffer. RU, resonance units.
to the multimeric ligand binding data returns the apparent rates of a surrogate monomeric ligand whose kinetics would generate approximately the same time courses as the multimeric ligand. The apparent on- and off-rates so obtained nevertheless reveal the much higher binding capacities of the IgG multimers than those of the IgG monomers. The apparent rates obtained from the multimeric ligand experiments are listed in Table 1.

Affinity of mAb CLBFcgran-1 Fab-sCD16a Interactions—It should be instructive to compare receptor-ligand and antibody-antigen interactions. CLBFcgran-1 Fab solutions at various concentrations were perfused over a sCD16a-derivitized surface. It is evident from Fig. 6A that CLBFcgran-1 Fab bound sCD16a with a much higher affinity and a much slower off-rate than any of the sCD16a ligands tested.

Antibody typically binds to antigen with a rather high on-rate. In such a case mass transfer may be the limiting step (4); fitting a transient model to the association and dissociation time courses would likely yield erroneous results. To circumvent this problem, Scatchard analysis (Fig. 6B) was used to derive the binding affinity of CLBFcgran-1 Fab for sCD16a. The estimated equilibrium dissociation constant is in the order of nanomolar (Table 2). In comparison, the $K_D$ values for IgG-CD16a interactions are in the order of micromolar.

The sCD16a-IgG Binding Kinetics Were Not Affected by the Injection Time—The presence of a significant amount of multimers in the chromatographed IgG could give rise to a multieponential appearance in both association and dissociation sensograms. Such a diagnostic feature was not observed (e.g. Fig. 3). However, this effect could be amplified by the long injection time because multimeric ligands would continue to bind the receptors after the binding of monomeric ligands had achieved equilibrium (25). Also, due to their high avidity, multimers, even if only present in undetectable amounts in the monomer solution, might gradually replace the bound monomers, thus giving rise to an artifactually slow dissociation. The longer the injection time, the slower the dissociation, as the bound multimers would accumulate on the sensor surface over time (25).

To address these issues, chromatographed IgG of various concentrations were perfused over immobilized sCD16a in an experiment with long injection time and low flow rate (10 min and 5 μl/min, respectively) to amplify the multimeric effect, if any. Again, multieponential appearance was not observed (Fig. 7). The kinetic rates so obtained ($k_{on} = 6.47 \times 10^9 \text{M}^{-1} \text{s}^{-1}$ and $k_{off} = 4.52 \times 10^{-3} \text{s}^{-1}$ from the data exemplified in Fig. 7) were compared with those listed in Table 1, which were evaluated from experiments that minimize the multimeric effect by using a short injection time and fast flow rate (2.5 min and 30 μl/min, respectively). The lack of dependence of kinetic rates on the injection time and flow rate, as shown by the excellent agreement between the two sets of values, suggests a minimal (if any) role of the effect in question.

The sCD16a-IgG Binding Kinetics Were Not Affected by Reconcentrating the Chromatographed IgG—Due to their limited quantities, the fractions of chromatographed hlgG1 and hlgG3 were reconcentrated in a protein concentrator before kinetic measurements. It has been reported that in this step some monomeric ligands may form multimeric aggregates (which might escape detection by chromatography), which may in turn affect the subsequent kinetic measurement (25). To address this issue, chromatographed hlgG was reconcentrated to a nearly 2-fold higher concentration (0.7 mg/ml) than those normally used in kinetic measurements, which was then used in an experiment with long injection time and slow flow rate (10 min and 5 μl/min, respectively) to favor multimeric binding. Again, multieponential appearance was not observed (Fig. 8). The on- and off-rates ($6.12 \times 10^{3} \text{M}^{-1} \text{s}^{-1}$ and $4.56 \times 10^{-3} \text{s}^{-1}$, respectively) so obtained are similar to those measured from experiments using chromatographed hlgG without reconcentration and with short injection time and fast flow rate (Table 1). This suggests that reconcentrating the chromatographed IgG did not have much of an adverse effect on the kinetic measurement.

**TABLE 2**

| Ligand       | CD16a Mix | CD16b Mix | CD16b Mix | CD16b Mix |
|--------------|-----------|-----------|-----------|-----------|
| sCD16a       | 1.07 ± 0.16 (n = 5) | 1.86 ± 0.19 (n = 3) | 29.9 ± 3.2 (n = 3) | 36.4 ± 12.4 (n = 3) |
| hlgG (μM)    | 0.72 ± 0.02 (n = 6) | 1.37 ± 0.30 (n = 3) | 31.1 ± 9.5 (n = 3) | 53.0 ± 11.0 (n = 3) |
| CLBFcgran-1 Fab (μM) | 2.37 ± 0.07 (n = 2) | 4.59 ± 0.09 (n = 2) | 6.46 ± 0.76 (n = 2) | 12.1 ± 4.9 (n = 2) |

**FIGURE 6. Binding of CLBFcgran-1 Fab to sCD16a.** A. CLBFcgran-1 Fab at concentrations of 1.6, 3.1, 6.2, and 25 nm (progressively higher curves) was perfused over immobilized sCD16a at a flow rate of 5 μl/min for 10 min. B. Scatchard analysis of equilibrium binding. The amount of mAb bound to sCD16a was plotted against the ratio of the bound to the free mAb concentration. A straight line was fit to the data and the equilibrium dissociation constant of CLBFcgran-1 Fab for sCD16a was calculated from the reciprocal negative slope of the line. The experiment was repeated twice with similar results (Table 2). RU, resonance units.
Binding to Partially Blocked sCD16a Resulted in Similar Kinetic Rates as Unblocked sCD16a—To test whether the two binding sites of the dimeric sCD16a molecule would work cooperatively to produce higher binding and slower dissociation, the active sites of immobilized sCD16a were partially blocked by injection of a sub-saturating concentration of CLBFcgran-1 Fab to reduce the maximum ligand binding capacity by 70%. Monomeric hlgG solutions at various concentrations were then injected to the sensor chip, as shown in Fig. 9. The globally fitted on- and off-rates (6.41 × 10^3 M^-1 s^-1 and 4.43 × 10^-3 s^-1, respectively) are very similar to those obtained from experiments using unblocked sCD16a (Table 1), suggesting the lack of cooperation between the two binding sites of the dimeric sCD16a.

CD16a™ on CHO Cell Surface Had Similar Binding Kinetics and Affinities to sCD16a on Sensor Chip—Two sets of experiments were performed to test whether sCD16a-Ig coupled on sensor chip has binding characteristics comparable with CD16a™ anchored on the cell surface. In the first set, the binding kinetics of monomeric hlgG for the CHO cell CD16™ were measured by real time flow cytometry. Fig. 10A shows association time courses of CHO cells transfected to express CD16™ due to binding of four concentrations of chromatographed fluorescein isothiocyanate-conjugated hlgG1 (closed symbols). Fig. 10B shows the corresponding dissociation time courses. The low affinity of CD16™-hlgG1 binding (Kd in μM range) required submilligram per ml concentrations of hlgG1 to achieve appreciable binding, which resulted in high nonspecific MFI of B7-1GPI-expressing CHO cells. The nonspecific MFI was especially high in the association assays, which also increased with increasing hlgG1 concentration (open symbols in Fig. 10A). This was due to the presence of a high concentration of fluorescence molecules in the binding buffer, as their removal in the dissociation assay greatly reduced the nonspecific MFI signals, suggesting that such signals were not caused by hlgG1 binding that could last more than 10 s, the time for the first data point.

Specific binding curves were obtained by subtracting MFI of B7-1GPI-expressing CHO cells from that of CD16™-expressing CHO cells measured at matched hlgG1 concentrations at

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**FIGURE 7.** Lack of effect of long injection time and slow flow rate. Chromatographed hlgG at concentrations of 3.33 μM (top curve) and four serial 2x dilutions (progressively lower curves) was perfused over immobilized sCD16a at a flow rate of 5 μl/min for 600 s. Both association and dissociation phases are shown in the overlaid sensograms after subtraction of the baseline drift and the refraction index difference between protein samples and the running buffer. RU, resonance units.

**FIGURE 8.** Lack of effect of reconcentration of chromatographed IgG. Monomeric hlgG after reconcentration in the protein concentrator to 4.56 μM (top curve) and four serial 2x dilutions (progressively lower curves) was perfused over immobilized sCD16a at a flow rate of 5 μl/min for 600 s. Both association and dissociation phases are shown in the overlaid sensograms after subtraction of the baseline drift and the refraction index difference between protein samples and the running buffer. RU, resonance units.

**FIGURE 9.** Binding to partially blocked sCD16a. sCD16a immobilized on the sensor chip was first blocked 70% by CLBFcgran-1 Fab. Monomeric hlgG at concentrations of 2.5 μM (top curve) and four serial 2x dilutions (progressively lower curves) was then perfused over the sensor chip at a flow rate of 30 μl/min for 150 s. Both association and dissociation phases are shown in the overlaid sensograms after subtraction of the baseline drift and the refraction index difference between protein samples and the running buffer. RU, resonance units.
matched time points (Fig. 10, C and D). Equations 1 and 2 were simultaneously fit (curves) to the corresponding association (Fig. 10C) and dissociation (Fig. 10D) data (points) for each hlgG1 concentration with a set of model parameters ($k_{on}$, $k_{off}$, $R_T$, and $\eta$). Unlike the fitting of SPR data, an additional parameter $\eta$ was introduced because the equilibrium MFI level from the association assay did not match the initial MFI level of the dissociation assay (Fig. 10, C and D). Similar to the analysis of SPR data, only the first 240 s in the dissociation data that follow single exponential decay were used in the fitting to minimize the potential impact of possible rebinding on the best-fit parameters. The individually fitted observed rate $k_{obs} = k_{on}L + k_{off}$ was found to increase linearly with the concentration of hlgG1 (Fig. 10E), as predicted by Equation 1. The $y$ axis intercept, the slope, and their ratio provide respective global estimates for off-rate ($k_{off} = 6.6 \times 10^{-3}$ s$^{-1}$), on-rate ($k_{on} = 2.6 \times 10^4$ M$^{-1}$ s$^{-1}$), and equilibrium dissociation constant ($K_d = 0.25$ $\mu$M), which are comparable with their averaged values from the four individual fits ($k_{off} = (3.20 \pm 0.19) \times 10^{-3}$ s$^{-1}$, $k_{on} = (3.41 \pm 0.54) \times 10^4$ M$^{-1}$ s$^{-1}$, $K_d = 0.10 \pm 0.02$ $\mu$M).

Experiments were performed at 0, 15, 20, and 37°C and the kinetic parameters so evaluated are plotted in Fig. 11. It is evident that they are comparable with those of the sCD16a-hlgG1 binding measured by SPR (Table 1). The standard free energy of binding at these temperatures were also calculated (Fig. 11D). The dependence of $\Delta G^0$ on $T$ was found to agree qualitatively with published results (14, 26).

To obtain independent confirmation of some of the above results, the affinities of CLBFcgran-1 Fab and monomeric IgG ligands for CHO cell surface CD16 isoforms were measured in the second set of experiments. Fig. 12 shows Scatchard analyses of the binding of $^{125}$I-labeled CLBFcgran-1 Fab to CD16a$^{TM}$, CD16b$^{NA2}$, and CD16b$^{NA1}$, and the estimated affinities are listed in Table 2. Fig. 13 shows the displacement curves of monomeric hlgG and RblgG for three CD16 membrane isoforms. The affinities, estimated from fitting Equation 3 to the data (6), are summarized in Table 2. It is evident that for all three cases tested (CLBFcgran-1 Fab, RblgG, and hlgG), the affinities of CD16a$^{TM}$ expressed on CHO cells are in reasonable agreement with those of sCD16a immobi-
lized on the BIAcore sensor chip. It should also be noted that CD16aTM bound hIgG and RbIgG with much higher affinities than did CD16bNA1 and CD16bNA2. CD16bNA1 and CD16bNA2 have similar affinities for hIgG and RbIGG.

**DISCUSSION**

The primary goal of the present work was to determine the kinetic rates of IgG-sCD16a interactions in solution. Whereas the increasingly popular SPR technology makes these measurements seemingly easy, careful studies have revealed several sources of potential artifacts that may be equally easily produced by the misuse of this technique (27). We therefore felt obliged to ask: are the kinetic rates measured in the present work intrinsic to monomeric IgG-sCD16a interactions? To address this question rigorously, several tests were performed to systematically rule out potential artifacts in SPR measurements. Possible sources of the artifacts include mass transport limitation (27), the presence of multimers due to reaggregation of the monomers either spontaneously or during the process of re-concentration (25), the cooperation between the two binding sites of the dimeric sCD16a, the use of inappropriate kinetic model, heterogeneity of the reactants, and problems in receptor immobilization (27). These are discussed below.

**Mass Transport Was Not Limiting in the IgG-sCD16a Interactions**—Otherwise, the association phase would have been slowed down by the limited supply of ligands and rebinding would have been significant during the dissociation phase, leading to underestimated off-rates. The criterion for a transport-limited reaction in the SPR measurement is the ratio of rate of mass transport to that of intrinsic molecular reaction, such that when $Q / R_{max} > 1$, the reaction is not considered to be transport-limited. $Q = mtc / k_{on} R_{max}$, where $R_{max}$ is the maximum ligand binding capacity of the receptor-coated surface and $mtc$ is the mass transport coefficient. Based on the analysis of convective and diffusive mass transport for the following, $mtc = 0.98(10^{-11}(294MW^{-1/3} - 0.7)/h)^{2/3} (f/0.3b)^{1/3}$

(Eq. 4)

where $b$, $l$, and $h$ are the respective width, length, and height of the flow cell, $f$ is the flow rate, and $MW$ is the molecular weight of the ligand. To examine whether the reactions in our experiments were transport limited, the $Q$ values were calculated and listed in Table 3. As can be seen, even at the low flow rate, the $Q$ values were 1–2 orders of magnitude greater than unity for all

![Figure 11](image_url1)  
**Figure 11.** Temperature dependence of CD16aTM-hIgG1 binding. The kinetic on-rate (A), off-rate (B), equilibrium dissociation constant (C), and standard free energy of binding (D) of CD16aTM-hIgG1 binding were plotted against temperature at which they were measured by real-time flow cytometry. Data are presented as mean ± S.E. estimated from two to four curves.

![Figure 12](image_url2)  
**Figure 12.** Scatchard analysis of CLBFcgran-1 Fab binding to isoforms of CD16 on CHO cells. The binding of varying concentrations of 125I-labeled CLBFcgran1 Fab to CD16aTM, CD16bNA1, and CD16bNA2 on the CHO cell surface was determined by radioimmunoassay. Specific CLBFcgran-1 Fab-CD16 binding was obtained by subtracting from the total binding to CD16-expressing CHO cells the background binding of the same concentrations of mAb to plain CHO cells. The concentration of the specifically bound mAb divided by that of the free mAb was plotted against the concentration of the bound mAb. A straight line was fit to each set of data (points) and the equilibrium dissociation constant of mAb for CD16 was calculated from the negative reciprocal slope of the line (Table 2). The experiment was done in triplicate and repeated twice with similar results.
The Kinetic Rates of Monomeric IgG-sCD16a Interactions Were Not Affected by the Possible Presence of Multimers— Gel filtration was used to separate monomers and multimers from commercial IgG. Had the chromatographed IgG reaggregated quickly, however, the monomeric IgG used in SPR measurement might have contained multimers of a significant amount to slow down the dissociation and led to underestimated off-rates. To test this possibility, monomeric IgG diluted by chromatography was re-concentrated to 5 mg/ml (~7 times higher than the highest concentration used in the SPR measurement) and passed through the gel filtration column again in the time scale of the SPR experiment (~4 h). As can be seen from Fig. 2A, the reconstituted IgG eluted as a single symmetric peak at the same position as that of the monomeric IgG in the previous gel filtration experiment. Thus, multimers were not detected in the second gel filtration. This result is in agreement with a previous report, which showed that monomeric IgG remained monomeric in solution for at least 48 h (12). Despite this evidence, low IgG concentrations (≤0.7 mg/ml) were nevertheless used in the SPR experiments, which were performed immediately after gel filtration. These precautions ensured that the amount of re-aggregated IgG (if any) would always be below the detection limit of size exclusion chromatography.

To test whether the undetectable trace amount of multimeric ligands (if any) would result in artifically slow apparent off-rates, SPR experiments were performed with different flow rates and injection times. It has been reported that when monomeric ligands were contaminated with multimers, longer injection time would result in more multimeric binding (25). Using multimeric ligands, we also found greater binding of larger-sized aggregated IgG with longer injection times (data not shown). However, a 4-fold prolonged injection time did not cause any change in the kinetic rates of monomeric IgG-sCD16a binding (Fig. 7). The same result was found even when reconstituted IgG was used (Fig. 8). Thus, either the kinetic rates were the same for both multimeric and monomeric interactions, or the multimer contamination was too high or too low for the kinetic rates to be sensitive to the further accumulation of multimers over time. The first two possibilities can be ruled out. Kinetic measurements of multimeric binding revealed their faster on-rates and slower off-rates (Table 1). Gel filtration experiments were unable to detect any multimer contamination in the monomer solution (Fig. 2A). Thus, even if trace amounts of multimers had existed in the chromatographed IgG, its concentration would have been too low to affect the kinetic rates of the monomeric IgG-sCD16a interactions. The lack of influence of aggregated ligands in our system is probably due to the low concentrations of monomeric ligands used in the SPR experiments (≤0.7 mg/ml). In the experiment of Maenaka et al. (14), by comparison, the ligand concentrations used were as high as 11.5 mg/ml.

Dimeric sCD16a Likely Has Similar Ligand Binding Kinetics as Monomeric Molecule—It is possible that two IgG molecules that are bound to the same sCD16a dimer associate to form a stable complex, which delays dissociation. Also, the dimeric sCD16a molecule may favor rebinding because there are two sites available for capturing the dissociated IgG instead of one, again resulting in an apparently slow off-rate. To address this
Ligand Binding Kinetics of CD16

issue, sCD16a was partially blocked in an experiment by a sub-saturating concentration of Fab fragment of CLBFcgr-1, an anti-CD16 adhesion blockade mAb (Fig. 9), which binds to an epitope near the ligand binding site (28). Assuming equal and independent binding of CLBFcgr-1 Fab to every antigen epitope regardless of whether the other epitope on the same sCD16a dimer is free or occupied, it can be estimated that when 70% of Fc binding sites was blocked, only 18% of those sCD16a dimers that have at least one site unblocked would have both sites available for IgG binding. In other words, >82% of functional sCD16a molecules only have a single site for IgG binding. Should the dimeric sCD16a cause slow dissociation, a much faster off-rate would have been predicted in this partially blocked configuration. That the kinetic rates in this experiment were found very similar to those measured using unblocked sCD16a dimers is free or occupied, it can be estimated that when 70% of Fc binding sites was blocked, only <18% of those sCD16a dimers that have at least one site unblocked would have both sites available for IgG binding. In other words, >82% of functional sCD16a molecules only have a single site for IgG binding. Should the dimeric sCD16a cause slow dissociation, a much faster off-rate would have been predicted in this partially blocked configuration. That the kinetic rates in this experiment were found very similar to those measured using unblocked sCD16a (k_{on} = 6.41 and 6.51 × 10^3 M^{-1} s^{-1} and k_{off} = 4.43 and 4.71 × 10^{-3} s^{-1} for experiments using partially blocked and unblocked sCD16a, respectively) suggests that the dimeric nature of sCD16a did not alter the kinetics of its monovalent interaction with ligands.

Binding Stoichiometry, Kinetic Mechanism, Receptor Orientation, Decaying Surface, and Ligand Heterogeneity—The present work used a Langmuir model to fit all the SPR data to estimate kinetic rates. The 1:1 stoichiometry for the IgG-sCD16a interaction is supported by a previous analytical centrifugation measurement (26) and by a hlgG1-sCD16 co-crystal structural model (29, 30). As possible kinetic mechanisms, we tested both a single-step model, A + B = C, and a two-step model, A + B = AB = C, where A, B, AB, and C denote, respectively, IgG, sCD16a, intermediate IgG-sCD16a complex, and stable IgG-sCD16a complex. Although using the two-step model resulted in slightly better fits to the data, the improvement was too small and the data were insufficient to warrant the more complicated model with two more freely adjustable parameters.

The presentation of surface molecule is known to affect SPR measurements; but the two-step procedure with capturing antibodies likely resulted in proper and uniform orientation of the immobilized sCD16a for ligand binding. However, immobilizing sCD16a this way yielded a decaying surface for ligand binding, because over time sCD16a dissociated from 214.1, which also dissociated from the rabbit anti-mouse Fc antibody. Although more rigorous treatment of the decaying surface is possible (31), the much slower dissociation of antibody-antigen interactions than that of IgG-sCD16a interactions allows simply subtracting the negatively drifting baseline to be an adequate correction for the effect of the antibody-antigen dissociation on the measured kinetic rates of the IgG-sCD16a binding.

The IgG ligands tested in the present work are heterogeneous even for those of single isotypes. For example, hlgG3 comprises at least 12 allotypes (24). Ligand heterogeneity appeared to affect the ability of the Langmuir model to fit the data of hlgG3 but not hlgG1, RbIgG, or hlgG. Total human IgG in serum is known to consist of four isotypes, with relative abundance of 60, 30, 6, and 3% for hlgG1 – 4, respectively (32). Preliminary experiments suggest that hlgG2 binds sCD16a with an affinity 15–20 times lower than that of hlgG1 (data not shown). The predominance of hlgG1 may explain why the Langmuir model appears to work in the case of hlgG despite its heterogeneity.

Comparison with Other Experiments—Having ascertained that intrinsic monomeric IgG-sCD16a interactions have indeed been measured, the next questions are, does sCD16a retain the binding characteristics of cell surface CD16a? How are our results compared with results from other studies? These are legitimate questions because sCD16a, which was made by fusing the extracellular domain of CD16a with the Fc fragment of hlG1 (17), lacks the transmembrane and cytoplasmic domains and the associated γ and/or ζ signaling subunits. Furthermore, the secreted sCD16a was purified by affinity chromatography and then immobilized on the biosensor chip via nonblocking antibody. Both the membrane anchor (6) and the γ chain (10) have been shown to alter kinetic rates/binding affinity.

To address the first question, two sets of independent binding studies were performed using CD16-expressing CHO cells. Real-time flow cytometry kinetic analysis yielded off-rates very similar to that measured by SPR, whereas the on-rates and hence dissociation constant from the two experiments differed by a few fold. Scatchard analysis showed that CLBFcgr-1 Fab bound to CHO cell CD16a_TM with a K_d ~ 2-fold of the value estimated in the SPR measurement using sCD16a. The K_d values of CD16a_TM for monomeric hlG and RbIgG measured from the competitive inhibition binding experiments were also ~2-fold of those of the sCD16a for the respective ligands via SPR measurement. These data are consistent with the results of a separate study of ours, which showed that the effect of CD16 anchor on its on-rate (but not the off-rate) depended on the nature of the anchor regardless of whether CD16 was on the cell surface or extracted from the cell and then immobilized on a surface. Given the different conditions between the CHO cell CD16a_TM experiment and the sCD16a experiment, it is difficult to further interpret the small differences in the binding parameters. Thus, sCD16a on the biosensor chip bound IgG ligands and a mAb with affinities and/or off-rate similar to those of CD16a_TM on the CHO cell membrane.

A previous SPR study of hlgG1 and hlgG3 binding to sCD16bNA2 (a monomer) reported 2–5-fold slower off-rates (0.98 and 2.63 × 10^{-3} s^{-1}, respectively) and similar (0.76 μM for hlgG1) or 7-fold larger (3.8 μM for hlgG3) equilibrium dissociation constants (13) compared with the values found here for the hlgG1- and hlgG3-sCD16a interactions (Table 1). By comparison, our competitive inhibition binding experiments using chromatographed ligands found that CHO cells CD16bNA1 and CD16bNA2 bound IgG (from both human and rabbit species) with K_d values several tens fold greater than those of CHO cell CD16a_TM (Table 2). On the other hand, a more recent SPR study using an inverted configuration (i.e. perfusing monomeric sCD16bNA2 over immobilized IgG) found much faster off-rates (half-life of a few seconds) (14). No resolution has been found at this point, and further studies are required to address these discrepancies.

We previously measured the kinetic rates of CHO cell CD16a_TM and CD16a_SPI, and CHO cell and K562 cell CD16bNA2 interacting with IgG coupled to the red blood cell surface using a micropipette technique (6–9). The off-rates

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N. Jiang, W. Chen, P. Selvaraj, and C. Zhu, unpublished data.
for the hlgG-CD16a<sup>TM</sup> and RblgG-CD16a<sup>TM</sup> (on CHO cells) and hlgG1-CD16b<sup>NA2</sup> (on CHO and K562 cells) interactions were 0.34, 0.24, 0.70, and 0.50 s<sup>-1</sup>, respectively. Although the solution (i.e. the so-called three-dimensional interaction) and surface (i.e. the so-called two-dimensional interaction) binding off-rates have the same unit of s<sup>-1</sup>, they have been theorized as physically distinct quantities (33). A major physical difference between the two- and three-dimensional binding is that, instead of approaching one another by free diffusion in the three-dimensional case, molecules in two-dimensional interactions are brought together (and apart) by cells to which they are anchored. Cells are 1,000 times the size of the molecules; and thereby their motions dictate the transport of the reacting molecules prior (and post) to their intrinsic binding (34, 35). The difference between the three-dimensional $k_{off}$ values measured via the SPR technique and the two-dimensional $k_{off}$ values measured by the micropipette technique has been highlighted in a recent study (36). The present study has provided another such example. These examples provide a starting point for studying the relationship between two- and three-dimensional kinetic parameters.

**REFERENCES**

1. van de Winkel, J. G. J., and Capel, P. J. A. (eds) (1996) Human IgG Fc receptors, R. G. Landes, Austin, TX.
2. Matsui, K., Boniface, J. J., Steffner, P., Reay, P. A., and Davis, M. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12862–12866.
3. McKeithan, T. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1553–1572.
4. Williams, T. E., Selvaraj, P., and Zhu, C. (2000) Biophys. J. 79, 1553–1572.
5. Chesa, S. E., Li, P., Nagarajan, S., Selvaraj, P., and Zhu, C. (2000) J. Biol. Chem. 275, 10235–10246.
6. Williams, T. E., Selvaraj, P., and Zhu, C. (2000) Biophys. J. 79, 1553–1572.
7. Williams, T. E., Nagarajan, S., Selvaraj, P., and Zhu, C. (2000) Biochem. J. 279, 1858–1866.
8. Huang, J., Chen, J., Chesa, S. E., Yago, T., Mehta, P., McEver, R. P., Zhu, C., and Long, M. (2004) J. Biol. Chem. 279, 44915–44923.
9. Miller, K. L., Duchemin, A. M., and Anderson, C. L. (1996) J. Exp. Med. 183, 2227–2233.
10. Sondermann, P., Jacob, U., Kutscher, C., and Frey, J. (1999) Biochemistry 38, 8469–8477.
11. Vance, B. A., Huizinga, T. W., Wardwell, K., and Guyre, P. M. (1993) J. Immunol. 151, 6429–6439.
12. Galon, J., Robertson, M. W., Galinha, A., Mazieres, N., Spagnoli, R., Fridman, W. H., and Sautes, C. (1997) Eur. J. Immunol. 27, 1928–1932.
13. Maenaka, K., van der Merwe, P. A., Stuart, D. I., Jones, E. Y., and Sondermann, P. (2001) J. Biol. Chem. 276, 44898–44904.
14. Nagarajan, S., Chesa, S., Coberin, L., Anderson, P., Zhu, C., and Selvaraj, P. (1995) J. Biol. Chem. 270, 25762–25770.
15. McHugh, R. S., Ahmed, S. N., Wang, Y. C., Sell, K. W., and Selvaraj, P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8059–8063.
16. Li, P., Nagarajan, S., Zhu, C., and Selvaraj, P. (2002) Mol. Immunol. 38, 527–538.
17. Chappel, M. S., Isenman, D. E. M., Xu, Y. Y., Dorrington, K. J., and Klein, M. H. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9036–9040.
18. Chappel, M. S., Isenman, D. E., Oomen, R., Xu, Y. Y., and Klein, M. H. (1993) J. Biol. Chem. 268, 25124–25131.
19. Selvaraj, P., Rosse, W. F., Silber, R., and Springer, T. A. (1988) Nature 333, 565–567.
20. Andrews, P. (1965) Biochem. J. 96, 595–606.
21. Morton, T. A., and Myszka, D. G. (1997) Methods Enzymol. 295, 268–294.
22. Allansmith, M., McClellan, B. H., Butterworth, M., and Maloney, J. R. (1998) J. Pediatr. 127, 278–290.
23. Propert, D. (1995) Exp. Clin. Immunogenet. 12, 198–205.
24. van der Merwe, P. A., Brown, M. H., Davis, S. J., and Barclay, A. N. (1993) EMBO J. 12, 4945–4954.
25. Ghirlanda, R., Keown, M. B., Mackay, G. A., Lewis, M. S., Unkeless, J. C., and Gould, H. J. (1995) Biochemistry 34, 13320–13327.
26. Schuck, P. (1997) Annu. Rev. Biophys. Biomol. Struct. 26, 541–566.
27. Tamm, A., and Schmidt, R. E. (1996) J. Immunol. 157, 1576–1581.
28. Sondermann, P., Huber, R., Oosthuizen, V., and Jacob, U. (2000) Nature 406, 267–273.
29. Radaev, S., Motyka, S., Fridman, W. H., Sautes-Fridman, C., and Sun, P. D. (2001) J. Biol. Chem. 276, 16469–16477.
30. Joss, L., Morton, T. A., and Myszka, D. G. (1998) Anal. Biochem. 261, 203–210.
31. Fridman, W. H., and Sautes, C. (1997) Cell-mediated Effects of Immunoglobulins, R. G. Landes, Austin, TX.
32. Bell, G. I. (1978) Science 200, 618–627.
33. Piper, J. W., Swerlick, R. A., and Zhu, C. (1998) J. Biol. Chem. 273, 492–513.
34. Chang, K. C., and Hammer, D. A. (1999) J. Biol. Chem. 274, 1280–1292.
35. Zhang, F., Marcus, W. D., Goyal, N. H., Selvaraj, P., Springer, T. A., and Zhu, C. (2005) J. Biol. Chem. 280, 42207–42218.