Differential Binding of Histidine-rich Glycoprotein (HRG) to Human IgG Subclasses and IgG Molecules Containing \( \kappa \) and \( \lambda \) Light Chains*  

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In previous studies we showed that the plasma protein histidine-rich glycoprotein (HRG) binds strongly to pooled human IgG. In the present work myeloma proteins consisting of different human IgG subclasses were examined for their ability to interact with human HRG. Using an IAsys optical biosensor we found initially that IgG subclasses differ substantially in their affinity of interaction with HRG. However, the most striking finding was the observation that the kinetics of the HRG interaction was dramatically affected by whether the IgG subclasses contained the \( \kappa \) or \( \lambda \) light (L)-chains. Thus, the on-rate for the binding of HRG to the \( \kappa \)-chain containing IgG1 and IgG2 (IgG1\( \kappa \) and IgG2\( \kappa \)) was \(-4\) and \(-10\)-fold faster than that for the binding of HRG to \( \lambda \)-chain containing IgG1 and IgG2 (IgG1\( \lambda \) and IgG2\( \lambda \)), respectively, with the dissociation constants (\( K_d \)) in the range 3–5 nm and 112–189 nm for the \( \kappa \) and \( \lambda \) isoforms, respectively. In contrast, the on-rate for the binding of HRG to IgG3\( \kappa \) and IgG4\( \kappa \) was found to be 9- and 20-fold slower than that for the binding of HRG to IgG3\( \lambda \) and IgG4\( \lambda \), respectively, with the \( K_d \) in the range 147–268 nm and 96–109 nm for the \( \kappa \) and \( \lambda \) isoforms, respectively. The binding of HRG to immunoglobulins containing the \( \kappa \)-chain (particularly IgG1\( \kappa \)) was generally potentiated in the presence of a physiological concentration (20 \( \mu \)M) of \( \text{Zn}^{2+} \) (\( K_d \) decreased to 0.60 \pm 0.01 for IgG1\( \kappa \)), but \( \text{Zn}^{2+} \) had no effect or slightly inhibited the binding of HRG to immobilized IgG subclasses possessing the \( \lambda \)-chain. Interestingly, HRG also bound differentially to Bence Jones (BJ) proteins containing \( \kappa \) and \( \lambda \)-chains, with HRG having a 14-fold lower \( K_d \) for BJ\( \kappa \) than for BJ\( \lambda \) when 20 \( \mu \)M \( \text{Zn}^{2+} \) was present. HRG also bound to IgM (IgMs), but the affinity of this interaction (\( K_d \) \(-1.99 \pm 0.05 \) pm) was markedly lower than the interaction with IgG, and the affinity was actually decreased 4-fold in the presence of \( \text{Zn}^{2+} \). The results demonstrate that both the heavy (H)- and L-chain type have a profound effect on the binding of HRG to different IgG subclasses and provide the first evidence of a functional difference between the \( \kappa \) and \( \lambda \)-chains of immunoglobulins.

Histidine-rich glycoprotein (HRG)\(^1\) is a 75-kDa protein that exists at relatively high levels in the plasma of many vertebrate species including humans (1) and has been the subject of investigation by a number of laboratories. Although the exact physiological function of HRG is unknown, a number of ligands for the molecule have been identified; these include hem (2), divalent metal ions (2), heparin (3, 4), heparan sulfate (5), plasminogen (6, 7), fibrinogen (8), and thrombospondin (9). HRG has been proposed to have a modular structure allowing it to bind several ligands independently (10), and evidence suggests that the interaction of HRG with glycosaminoglycans is regulated by metal ions and pH (11). HRG also has been found to be a regulator of heparin binding growth factor action (5), lymphocyte proliferation (12), and lymphocyte cell adhesion (13–15). Recently, we showed that HRG binds strongly to immunoglobulin (IgG) and inhibits the formation of insoluble immune complexes (IICs) between ovalbumin and polyclonal rabbit anti-ovalbumin IgG in vitro (16). These latter findings have implicated HRG in regulating IIC formation in vivo, particularly in relation to patient suffering from autoimmune diseases like rheumatoid arthritis and systemic lupus erythematosus, where there is an excessive production of autoantibodies and the formation of IICs (17–19). The failure of IICs to be cleared from the blood circulation in these patients may result in the deposition of IICs in specific target tissues, and this may be an important factor in the pathogenesis of these diseases (20). The associated pathology may be caused, at least in part, by the ability of the deposited IICs to activate the complement pathway and to induce inflammation in the target tissue and/or by tissue injury resulting from decreased nutrient transport (21).

Immunoglobulin molecules consist of two identical heavy (H)- and light (L)-chains, with the variable regions of the H- and L-chains associating to form the antigen binding site of the antibody. There are nine types of H-chains in humans (\( \mu, \delta, \gamma_1, \gamma_2, \beta_2, \alpha_1, \alpha_2, \) and \( \epsilon \)) that define a range of classes and subclasses. The functional role of each of the H-chains, with the exception of the \( \delta \)-chain, is well characterized (22). In addition, in many species there are two types of L-chains, termed \( \kappa \) and \( \lambda \), but unlike the different H-chain types, their function is unknown (22). Our recent finding that HRG binds to human IgG with high affinity and inhibits the formation of IICs (16) has raised the question of whether HRG binds with the same affinity to the different IgG subclasses (which can possess either \( \kappa \) or \( \lambda \)-L-chains). In the present study we use optical biosensor techniques to study the binding of HRG to several different subclasses of IgG and also to IgM. Our results show that HRG has a differing affinity for the various IgG subclasses.

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\( ^{1} \) The abbreviations used are: HRG, histidine-rich glycoprotein; BJ, Bence Jones proteins; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; IC, immune complex; IIC, insoluble immune complex; \( K_{d} \), dissociation constant; STP, streptavidin; T, Tween-20; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; PBS, phosphate-buffered saline; NHS, N-hydroxysuccinimide; NHS-LC-biotin, sulfo-L-cysteine-6-(biotinamido) hexanone; b-, biotinylated.

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but that the L-chain type of IgG also has a marked effect on HRG binding. Thus, the affinity of HRG for the x forms of IgG1 and IgG2 is at least 10-fold greater than for the A forms of IgG1 and IgG2 (and other IgG subclasses), with these interactions being significantly affected by the presence of Zn2+ . The results provide strong evidence that the binding of HRG to IgG molecules is profoundly influenced by both the H- and L-chain isotype of the IgG molecule, an observation that may have relevance to the ability of HRG to inhibit the insolublization of IgG-containing immune complexes (IC).

MATERIALS AND METHODS

Reagents—Human myeloma IgG1, IgG1A, IgG2A, IgG2B, IgG3k, IgG3λ, and IgG4k, were purchased from Sigma; IgG4A and IgMx were from Calbiochem-Novabiochem and Accurate Chemicals, respectively. Bence Jones (BJ) proteins were a generous gift from Dr. Bob Raison, University of Technology, Sydney. Human IgG (isolated from pooled human serum), bovine serum albumin (BSA, fraction V), aprotinin, polyoxymethyleneosorbin monolaurate (Tween 20), phosphate-citrate buffer with sodium phosphate parasorbates and 2.2’-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) diammonium (ABTS) were purchased from Sigma. Carboxymethyl dextran cuvettes for the IAsys biosensor, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), dimethylaminopropylamine were purchased from Fisons Affinity Sensors, Cambridge, UK. Streptavidin (STP) was purchased from Pro- tein Industries, Australia. Sulfosuccinimidyl 6-(biotinamido) hexanooate (NHS-LC-biotin), gentle Ag/Ab elution buffer (a cuvette regeneration buffer gentle to the dextran matrix), and horseradish peroxidase (HRP)-conjugated STP (HRP-STP), were purchased from Pierce.

Purification of Native Human HRG—Native human HRG of 75-kDa molecular mass was purified from fresh human plasma as described previously (23) by equilibrating a phosphocellulose column with loading buffer containing 10 mM sodium phosphate (pH 6.8) that contained 1 mCi EDTA and 0.5 mM NaCl. The plasma was mixed with EDTA and NaCl to final concentrations similar to the loading buffer and with 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) (ICN Pharmaceutical Inc., Costa Mesa, CA) at 100 μg/ml and aprotinin (a tryspin inhibitor) at 2 μg/ml. The plasma was passed through the equilibrated column, and unbound protein was removed by extensive washing of the column with the loading buffer. Bound HRG was then eluted from the column using the same buffer containing 2 M NaCl.

Biotinylation of Proteins—The proteins to be biotinylated (e.g. IgG subclasses, HRG, IgM, and BJ proteins) were dissolved in PBS. SDS-polyacrylamide gel electrophoresis analysis showed that BJx consisted of two bands of molecular mass ~22 kDa and ~44 kDa, whereas the BJx consisted of only a single band of molecular mass ~44 kDa. These proteins were subjected to gel filtration using a Superose 12 fast protein liquid chromatography column to separate the monomeric from the dimeric form of each protein, particularly for BJx; BJλ showed only a single peak, demonstrating that it existed exclusively in a dimeric form. The dimeric form of each protein was biotinylated for use in all ELISA and biosensor studies.

Binding of IgG Subclasses, IgM, and BJ Proteins to Immobilized HRG—The binding of biotinylated forms of human IgG subclasses (myeloma-derived), IgM, and BJ proteins to immobilized human HRG was examined by ELISA. HRG (20 μg/ml) was immobilized on Maxisorb ELISA trays by incubating in 0.1 M NaHCO3 buffer (pH 9.6) for 1 h at 37 °C. The trays were blocked with PBS containing 10 mg/ml BSA and 3 mM NaNO2 (PBS-BSA-Az) for 1 h at 37 °C and then incubated with biotinylated proteins in the concentration range between 1.6 and 100 μg/ml in PBS-BSA-Az for 3 h at 37 °C. Unbound proteins were washed away three times with PBS containing 10 mg/ml BSA and 0.05% Tween 20 (PBS-BSA-T). Bound biotinylated proteins were detected by incubating the trays with HRP-STP (in 1/500 dilutions) in PBS containing 10 mg/ml BSA (PBS-BSA) for 1 h at 37 °C. This was followed by incubating the trays with the colorimetric HRP substrate ABTS (0.2 mg/ml) for 30 min at 37 °C in a 0.1 M sodium citrate, 0.1 M sodium phosphate buffer (pH 5.0) containing 0.03% (v/v) sodium perborate (a substitute for hydrogen peroxide). The absorbance of the solution of each well was measured at 405 and 490 nm using an ELISA plate reader (dual wavelength measurement). The results showed that the binding of these proteins to immobilized HRG was concentration-dependent. The HRG color development was linear for at least 3 h, and the trays coated with BSA instead of HRG or when the trays were incubated with biotinylated BSA (b-BSA) instead of the biotinylated proteins. These control experiments, therefore, indicated that the binding of these proteins was specific for HRG.

Determination of Binding Constants Using the Biosensor—An IAsys reagent (for biosensor studies, Carlsberg, Denmark) with a carboxymethyl dextran-sensing cuvette was used to determine the kinetic constants and affinities of the binding of HRG to immobilized ligands (e.g. IgG, IgM, and BJ proteins). Except where indicated, all experiments were performed in PBS-BSA-T and at a temperature of 25 °C. The BSA (1%, v/v) was included in the buffer to reduce any nonspecific binding of the proteins and also to buffer the effects of any added Zn2+ (in the experiments where Zn2+ was included), since a large proportion of plasma Zn2+ is bound to albumin (26). The reaction vessel was stirred continuously by the aid of a propeller. Binding was measured at 2-s intervals, and the readout from the biosensor was in units of arc s. Each binding reaction was routinely followed for 5 min. All binding experiments were performed at least in duplicate. The Fast Fit program supplied by Fisons was used to evaluate the kinetic constants (27).

Coupling of STP to the Dextran Matrix—STP was coupled via ε-amino groups to the carboxymethyl dextran-sensing surface of the IAsys biosensor cuvette using EDC and NHS (24, 25). This was done by equilibrating the cuvette in PBS buffer containing 0.05% Tween 20 (PBS-T) and then reacting the cuvette with a mixture of EDC/NHS for 7 min. Unreacted EDC/NHS was washed away with PBS-T followed by three washes with 0.01 M sodium acetae buffer (pH 4.5). STP (50 μg/ml) in indicated acetate buffer was added to the cuvette and allowed to react with the activated carboxyl groups for 5 min. Uncoupled STP was removed by washing with the acetate buffer, and unreacted spectrin-like groups were blocked by incubating with ethanolamine (1 M, pH 8.5) for 2 min. The cuvette was washed three times with the acetate buffer and then washed with PBS-T followed by a wash with 10 mM HCl to remove any noncovalently bound protein. A biosensor response of ~500 arc s for the immobilized STP was observed. According to the data provided by the manufacturer this response represents the coupling of 3 ng of STP/mm2 of sensing surface.

Binding of Biotinylated Proteins to the Biosensor Surface—Biotinylated IgG subclasses, IgM, or BJ proteins were dissolved in PBS, and protein-ligand was then added in PBS-T at different concentrations close to the immunoglobulin binding region(s), and that this region(s) is sterically hindered upon immobilization of the HRG. Biosensor studies of the binding of HRG to immobilized proteins, therefore, could only be carried out by examining the binding of soluble HRG to immobilized immunoglobulins.

Binding of Protein Ligands to Proteins on the Biosensor Surface—Before immobilization of the biotinylated proteins on the STP-dextran, nonspecific binding between the protein ligands and the STP-dextran layer was reduced by the addition of different amounts of the nonbiotinylated protein ligands in PBS-BSA-T to the cuvette. Under these conditions no significant level of nonspecific binding of any of the protein ligands used in this study could be detected.

Preliminary experiments were performed to establish the concentration range of protein-ligand suitable for kinetic analysis. PBS-T was added to the protein-coupled cuvette to establish a baseline (5 min), and protein-ligand was then added in PBS-T at different concentra-
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tions. Binding of the protein-ligand was studied by monitoring the association phase for 5 min. Subsequently, the cuvette was washed with PBS-T, and the dissociation phase was monitored for 5 min. Bound protein-ligand was removed (cuvette regeneration) by washing with either 10 mM HCl or the gentle Ag/Ab elution buffer (Pierce). The cuvette was then washed after the washing of the cuvette with PBS-T. As previously (16), we found no evidence that the 10 mM HCl cuvette regeneration wash significantly affected the ability of the proteins to simultaneously interact with immobilized proteins on the cuvette surface.

Evaluation of the Kinetic Constants—The IAsys biosensor was provided with a digital DECPc 450D, LP computer. Data obtained with the biosensor were transferred directly to the Fast Fit program (Fisons Applied Sensor Technology). This program uses an iterative curve-fitting to derive the observed rate constant and the maximum response at equilibrium due to ligand binding at the particular ligand concentration. The association of a soluble ligand with an immobilized macromolecule can be described by the pseudo first order equation $R_t = R_0 + E + k_1(1 - e^{-k_{obs}t})$, when only one binding site is available for the ligand (24). In this equation, $R_t$ is the IAsys response at time $t$. Units of $E$ (this is proportional to the concentration of ligand-protein complex at time $t$, $R_t$ is the IAsys response at time $t = 0$ in units of ars induced by the addition of the ligand solution to the buffer in the cuvette (this represents a net displacement of the biosensor signal, and its value is determined by the Fast Fit program for each binding curve analyzed). $R_0$ is the maximum IAsys response due to binding of ligand at equilibrium ($E = 0$) and $k_{obs}$ is the observed rate constant (term $k_{obs}$ in Fast Fit) given by $k_{obs} = k_{on} / [ligand]$ (27). For two binding sites the Fast Fit program uses the equation $R_t = R_0 + E_1(1 - e^{-k_{obs}t}) + E_2(1 - e^{-2k_{obs}t})$ to derive the kinetic constants (24). In this equation $E_1$ and $E_2$ are the maximum response at equilibrium due to binding of the ligand to the high and low affinity binding site, respectively; and $k_{obs}$ and $k_{on}$ represent the observed rate constants for the high and low affinity binding site, respectively. This equation assumes that, at equilibrium, the ligand-protein complex is stable; thus, the equilibrium line must be parallel to the starting baseline ([ligand-protein]), and at least 80–90% of the data must be taken into account when fitting data to a curve for either single or double exponential binding.

Kinetic Constants for HRG-IgG and HRG-IgM Interactions—The binding of HRG to L-chain containing IgG subclasses (IgGα) and IgM could only be fitted to a single exponential and not to a double exponential. A linear relationship was obtained by plotting $k_{obs}$ versus HRG concentration for the interaction of HRG with these IgG subclasses (IgGα) and IgM. In contrast, no linear relationship was obtained by plotting $k_{obs}$ versus HRG concentration for the interaction of HRG with immobilized human IgG subclasses containing $\kappa$-L-chain (IgGκ), indicating that the data does not fit an exponential curve. Therefore, in these instances, similar to other kinetic analyses (27), the first region of the progress curve that best fits the single exponential term (assuming that thermodynamic equilibrium is reached) was used to evaluate the parameters for the highest affinity interaction. This was done by plotting $E/E - (R_t - R_0)$ versus time and selecting the linear region of this plot.

In some instances the Fast Fit program was used to extrapolate the dissociation data to the base line for determination of the $k_{obs}$ parameter. Values of $K_d$ were obtained by using the relationship $K_d = k_{off} / k_{on}$ in good approximation with those obtained by Scatchard analysis of the extent of association (not shown).

RESULTS

Binding of HRG to IgG Subclasses Possessing $\kappa$-L-Chain—We have previously shown that HRG binds to immobilized human IgG (from pooled human serum) with high affinity ($K_d = 85 \pm 15$ nM) (16). To determine whether HRG binds with a similar affinity to each different IgG subclass, preliminary experiments were carried out to examine the binding of biotinylated IgGκ, IgG2κ, IgG3κ, and IgG4κ to immobilized HRG in an ELISA assay. In these experiments, which involved the incubation of HRP-STP conjugate followed by color development, the concentration of immunoassay was determined previously (16), the binding of each IgG subclass to the immobilized HRG could be detected. The experiments indicated that each of the four different subclasses of IgG binds to immobilized HRG in a concentration-dependent manner. Moreover, the dissociation constants ($K_d$) for the binding of IgG(κ) to immobilized HRG, as determined by Scatchard analysis after plotting (1/maximum bound) versus (1/IgG concentration), was found to follow the relationship $K_d < K_d < K_d < K_d$ (data not shown).

To further characterize the interaction of HRG with IgG subclasses, the binding of native human HRG to each of the four IgG subclasses possessing the $\kappa$-L-chain was also examined using the IAsys biosensor. As noted above, these studies required the immobilization of the IgG subclasses rather than the HRG, as the immobilization of the HRG to the biosensor surface resulted in the HRG losing its ability to interact with any of the immunoglobulins, probably due to steric effects. Therefore, for these studies 20–60 ng of biotinylated human IgG (of the indicated subclass possessing $\kappa$-L-chain) was immobilized via STP coupling onto the surface of a dextran cuvette, and the binding of soluble HRG to the immobilized IgG subclass was carried out following equilibration of the cuvette in PBS-BSA-T. Thus, HRG was added in PBS-BSA-T, and the association phase for the binding of native human HRG to the immobilized IgG1κ was monitored for 5 min. Subsequently, the cuvette was washed three times with PBS-BSA-T buffer (to bring the liquid phase HRG concentration to zero), and the dissociation phase was monitored for 5 min. The biosensor profiles for the binding of different concentrations of HRG to immobilized human IgG1κ are shown in Fig. 1A. The data show that the binding of HRG to IgG1κ is dependent on the HRG concentration in the range of 10–400 nM and that within this concentration range the binding of HRG is saturable with near-maximal binding (∼500 ars) occurring at a HRG concentration of 400 nM. The binding of HRG was significantly inhibited (>80%) when the HRG was preincubated before the addition to the cuvette with soluble IgG1κ (IgG1κ/HRG molar ratio > 4, data not shown), suggesting that the HRG interacted specifically with IgG1κ. The observed rate constant for each binding curve, at the indicated concentrations of HRG, was obtained by fitting the curve to the single exponential expression using the Fast Fit program as described previously (16). A plot of the observed rate constant ($k_{obs}$ s$^{-1}$) against the concentration of HRG (nm) approximated a straight line (see Fig. 1B), with the line of best fit revealing that HRG binds to immobilized IgGκ with an on-rate of 1.73 ± 0.04 × 10$^5$ M$^{-1}$ s$^{-1}$ (slope of the plot) and an off-rate of 0.53 ± 0.02 × 10$^{-3}$ s$^{-1}$ (intercept with the y axis) (see Table I). The dissociation constant for this interaction was determined either using the relationship $K_d = k_{off} / k_{on}$ or by Scatchard analysis of the maximum amount of bound HRG at equilibrium; both methods gave a $K_d$ of 3.0 ± 0.1 nM (see Table I).

Similar biosensor experiments carried out to study the interaction of soluble HRG with immobilized IgG2κ, IgG3κ, and IgG4κ (each immobilized on to separate biosensor cuvettes) also indicated that the binding of HRG to each of these subclasses was saturable and dependent on the HRG concentration. As previously, the biosensor profiles for the binding of HRG (at each concentration) to each IgG subclass was fitted to a single exponential to derive the $k_{obs}$ parameter. As shown in Fig. 2B and Table I, the on-rate, off-rate, and $K_d$ for the interaction of HRG with IgG2κ were found to be 2.8 ± 0.3 × 10$^5$ M$^{-1}$ s$^{-1}$, 1.4 ± 0.2 × 10$^{-3}$ s$^{-1}$, and 5.0 ± 1.3 nM, respectively. Interestingly, the off-rate for the interaction of HRG with IgG3κ was 0.13 ± 0.01 × 10$^5$ M$^{-1}$ s$^{-1}$ (see Fig. 2C) and that for HRG with IgG4κ was 0.11 ± 0.01 × 10$^5$ M$^{-1}$ s$^{-1}$ (see Fig. 2D); these values are ∼15–30-fold slower than those observed for IgG1κ or IgG2κ. Further analysis showed that the off-rate and $K_d$ for the interaction of HRG with immobilized IgG3κ were 1.9 ± 0.1 × 10$^{-3}$ s$^{-1}$ and 148 ± 13 nM, respectively. The corresponding values for IgG4κ were different: off-rate = 2.9 ± 0.2 × 10$^{-3}$ s$^{-1}$, and $K_d = 268 ± 27$ nM. Since the half-life ($t_{1/2}$) corresponding to the
TABLE I

| Interactions | $k_{on}$ | $k_{off}$ | $K_d$ |
|--------------|---------|---------|--------|
| HRG to immobilize IgG1k | $1.73 \pm 0.04$ | $0.53 \pm 0.02$ | $3.0 \pm 0.1$ |
| Control | $6.21 \pm 0.13$ | $0.37 \pm 0.17$ | $0.60 \pm 0.01$ |
| +20 μM Zn$^{2+}$ | $2.8 \pm 0.3$ | $1.4 \pm 0.2$ | $5.0 \pm 1.3$ |
| HRG to immobilize IgG2k | $3.4 \pm 0.4$ | $1.1 \pm 0.2$ | $3.3 \pm 0.1$ |
| Control | $0.13 \pm 0.01$ | $1.9 \pm 0.1$ | $148 \pm 13$ |
| +20 μM Zn$^{2+}$ | $0.20 \pm 0.01$ | $1.8 \pm 0.1$ | $92 \pm 9$ |
| HRG to immobilize IgG3k | $0.11 \pm 0.01$ | $2.9 \pm 0.2$ | $268 \pm 27$ |
| Control | $0.15 \pm 0.02$ | $2.5 \pm 0.1$ | $167 \pm 26$ |
| +20 μM Zn$^{2+}$ | $0.77 \pm 0.04$ | $2.6 \pm 0.3$ | $352 \pm 4.9$ |
| HRG to immobilize IgG4k | $1.30 \pm 0.04$ | $1.4 \pm 0.7$ | $10.4 \pm 1.2$ |
| Control | $0.036 \pm 0.005$ | $7.1 \pm 0.1$ | $1990 \pm 50$ |
| +20 μM Zn$^{2+}$ | $0.016 \pm 0.003$ | $11.8 \pm 1.6$ | $7752 \pm 1707$ |
| HRG to immobilize IgG1λ | $0.44 \pm 0.02$ | $8.1 \pm 2.4$ | $189 \pm 43$ |
| Control | $0.34 \pm 0.02$ | $9.1 \pm 3.1$ | $266 \pm 35$ |
| +20 μM Zn$^{2+}$ | $0.27 \pm 0.01$ | $3.1 \pm 0.2$ | $112 \pm 3$ |
| HRG to immobilize IgG2λ | $0.21 \pm 0.05$ | $3.1 \pm 0.4$ | $150 \pm 52$ |
| Control | $1.16 \pm 0.09$ | $12.2 \pm 2.1$ | $109 \pm 29$ |
| +20 μM Zn$^{2+}$ | $1.11 \pm 0.18$ | $13.2 \pm 1.6$ | $113 \pm 23$ |
| HRG to immobilize IgG3λ | $2.16 \pm 0.03$ | $20.8 \pm 0.8$ | $96 \pm 5$ |
| Control | $1.91 \pm 0.04$ | $19.8 \pm 1.9$ | $103 \pm 8$ |
| +20 μM Zn$^{2+}$ | $0.38 \pm 0.02$ | $2.90 \pm 0.04$ | $76 \pm 6$ |
| HRG to immobilize IgMk | $0.32 \pm 0.05$ | $4.5 \pm 0.1$ | $140 \pm 5$ |

* $p < 0.001$.  ** $p < 0.01 > 0.001$.  † $p < 0.05 > 0.01$.  ‡ $p < 0.01$.  § $p < 0.05 > 0.01$.  ¶ $p < 0.01 > 0.001$.

**Binding of HRG to IgG Subclasses Possessing l L-Chains**

The finding that HRG differs in its ability to interact with different subclasses of IgG containing the k L-chain suggested that differences also may exist in the ability of HRG to interact with subclasses of IgG containing the l L-chain. Therefore, to study the binding of HRG to different l L-chain-containing IgG subclasses, each subclass (IgG1k, IgG2k, IgG3k, and IgG4k) was immobilized onto a separate biosensor cuvette, and the binding of soluble HRG to each subclass was analyzed using the biosensor. The binding of HRG to each different IgG subclass was carried out separately at a range of different concentrations of HRG (10–600 nM) in PBS-BSA-T. The binding of HRG to each IgG subclass was found to be saturable and dependent on HRG concentration (data not shown). The biosensor read out for each concentration of HRG was fitted to a single exponential to obtain the average $k_{obs}$ for the interaction, and the average $k_{obs}$ was then plotted against the HRG concentration.

To facilitate comparison of the results from IgG subclasses containing either k or l L-chains, the data for each l-containing subclass was plotted on the same graph as the corresponding k-containing subclass; the results for IgG1k and IgG2k are shown in Fig. 2, A and B, and those for IgG3k and IgG4k are shown in Fig. 2, C and D. From the line of best fit it was calculated (Table I) that the on-rate for the interaction of HRG with IgG1k and IgG2k was $0.44 \pm 0.02 \times 10^5$ M$^{-1}$ s$^{-1}$ and $0.27 \pm 0.01 \times 10^5$ M$^{-1}$ s$^{-1}$, respectively; these values are...
bars represent each concentration of HRG.

m binding of human HRG to each of eight different IgG subclasses (IgG1, IgG2, IgG3, and IgG4) was examined using the IAsys biosensor. Each human IgG subclass was immobilized onto a separate biosensor cuvette, and then different concentrations of HRG were added, and the association and dissociation of HRG were monitored exactly as described in the legend to Fig. 1. The $k_{on}$ values for the binding of HRG to each IgG subclass was determined by fitting the biosensor data to a single exponential. The $K_d$ for the binding of HRG to each IgG subclass was calculated from the association and dissociation data (without affecting the off-rate of this interaction). As previously, under the conditions used in the analyses the measured on-rates are unlikely to be significantly affected by dissociation.

The results show that although the presence of Zn$^{2+}$ generally increases the affinity of the binding of HRG to all IgG subclasses possessing $\kappa$ L-chains, it tends to slightly decrease the affinity of the binding of HRG to IgG subclasses possessing $\lambda$ L-chains (see Table I). However, except for IgG1k, the effects of Zn$^{2+}$ on the HRG-IgG interaction were small and, in most cases, not statistically significant.

Binding of HRG to Bj Proteins—The above studies indicated that the L-chain isotype may be a major factor in determining the kinetics of the interaction between HRG and the different IgG subclasses. To further characterize the interaction of HRG with IgG, studies were therefore carried out using $\kappa$ and $\lambda$ L-chain-containing Bj proteins. For these studies, lyophilized Bj proteins were dissolved in PBS and purified by gel filtration on fast protein liquid chromatography using a Superose 12 column. Two protein peaks were obtained with the BjIs preparation, and a single higher molecular weight peak was obtained with BjA. Subsequently, SDS-polyacrylamide gel electrophoresis analysis of the protein peaks showed that BjIs consisted of a lower molecular weight monomeric and a higher molecular weight dimeric form, whereas only a single peak of the dimeric form of BjA could be detected (not shown). The dimeric forms of both BjIs and BjA were biotinylated as described under “Materials and Methods” and then used in ELISA assays to assess their ability to bind immobilized HRG. Preliminary experiments indicated that both biotinylated $\kappa$ and $\lambda$ Bj proteins bound to immobilized HRG in an ELISA assay, that the binding was concentration-dependent, and that HRG bound more strongly to the $\kappa$ than to the $\lambda$ L-chains (data not shown).

The interaction of HRG with Bj proteins also was studied using the biosensor. For these studies each Bj protein was immobilized onto a separate biosensor cuvette to permit an analysis of the binding of HRG in solution. The biosensor data indicated that the binding of HRG to both BjIs and BjA proteins was saturable and dependent on the HRG concentration. Similar to the analysis of other biosensor data described above,

5–10-fold slower than for the binding of HRG to IgG1k and IgG2k (Fig. 2, A and B). The off-rates and $K_d$ were found to be 8.1 ± 2.4 × 10$^{-1}$ s$^{-1}$ and 189 ± 43 nm for the HRG-IgG1k interaction and 3.1 ± 0.2 × 10$^{-3}$ s$^{-1}$ and 112 ± 3 nm for the HRG-IgG2k interaction, respectively. Interestingly, the dissociation constants were 20–60-fold higher than those observed for IgG1k and IgG2k (see Fig. 2 and Table I).

As shown in Fig. 2 and Table I, similar studies indicated that the binding of HRG to immobilized IgG3k and IgG4k is faster than the binding of HRG to IgG1k and IgG2k. The on-rate, off-rate, and $K_d$ for the IgG3k/IgG4k interaction were 1.16 ± 0.09 × 10$^5$ s$^{-1}$, 12.2 ± 2.1 × 10$^{-3}$ s$^{-1}$, and 190 ± 29 nm, respectively (Fig. 2C, Table I). The corresponding values for the IgG3k/IgG4k interaction were 2.16 ± 0.03 × 10$^5$ s$^{-1}$, 20.8 ± 0.8 × 10$^{-3}$ s$^{-1}$, and 96 ± 5 nm, respectively (Fig. 2D, Table I). The dissociation constant was decreased from 0.37 ± 0.17 × 10$^{-3}$ s$^{-1}$ to 0.037 ± 0.02 × 10$^{-3}$ s$^{-1}$ (see Table I).

Effect of Zn$^{2+}$ on the Binding of HRG to IgG Subclasses—Previously it was shown that the presence of Zn$^{2+}$ potentiates the binding of HRG to human IgG (from pooled human serum) but inhibits the binding of HRG to the complement component C1q (16). To determine the effect of Zn$^{2+}$ on the binding of HRG to IgG subclasses, biosensor experiments were performed using separate cuvettes containing each immobilized IgG subclass (as described above). The binding of HRG to each different IgG subclass was carried out in PBS-BSA-T containing 20 μM added Zn$^{2+}$ (PBS-BSA-T-Zn). As shown in Fig. 2A and Table I, in the presence of Zn$^{2+}$ the on-rate of the HRG-IgG1k interaction was increased from 1.73 ± 0.04 × 10$^5$ to 6.21 ± 0.13 × 10$^5$ M$^{-1}$ s$^{-1}$, whereas the off-rate was decreased from 0.53 ± 0.02 × 10$^{-3}$ to 0.37 ± 0.17 × 10$^{-3}$ s$^{-1}$. The dissociation constant was decreased ~5-fold, changing from 3.0 ± 0.1 to 0.60 ± 0.01 nm (see Table I).
for each concentration of HRG, the observed rate constant ($k_{obs}$) was obtained by fitting the binding curve to a single exponential using the Fast Fit program. Binding studies showed that the change of the $k_{obs}$ for the interaction of HRG with both Bjκ and Bjλ was dependent on the HRG concentration. The results (Table I) indicate that the on-rate, off-rate, and the $K_d$ for the binding of HRG to immobilized Bjκ are 0.77 ± 0.04 × 10^5 M⁻¹ s⁻¹, 2.6 ± 0.3 × 10⁻³ s⁻¹ and 35.2 ± 4.9 nM, respectively, and that the corresponding values for the binding of HRG to immobilized Bjλ are 0.38 ± 0.02 × 10^5 M⁻¹ s⁻¹, 2.90 ± 0.04 × 10⁻³ s⁻¹, and 76 ± 6 nM, respectively. As outlined above the measured on-rates are unlikely to be significantly affected by dissociation ($t_{1/2}$ ~ 4 min for the interaction of HRG with Bjκ and Bjλ). The results indicate, therefore, that the affinity for the binding of HRG to Bjκ is significantly higher than for the binding of HRG to Bjλ.

To determine whether Zn²⁺ affects the ability of HRG to interact with Bjκ and Bjλ experiments were carried out as described above, with the exception that the HRG was added to the cuvette in PBS-BSA-T-Zn. As shown in Table I, in the presence of Zn²⁺ the on-rate of the HRG-Bjκ interaction was increased from 0.77 ± 0.04 × 10^5 to 1.30 ± 0.04 × 10^5 M⁻¹ s⁻¹, and the off-rate was decreased from 2.6 ± 0.3 × 10⁻³ to 1.4 ± 0.7 × 10⁻³ s⁻¹. The $K_d$ was significantly reduced from 35.2 ± 4.9 to 10.4 ± 1.2 nM. In contrast, an investigation of the effect of Zn²⁺ on the HRG-Bjλ interaction indicated that the presence of Zn²⁺ only slightly decreased the on-rate of the interaction from 0.38 ± 0.02 × 10^5 to 0.32 ± 0.05 × 10^5 M⁻¹ s⁻¹ but significantly increased the off-rate of the interaction from 2.90 ± 0.04 × 10⁻³ to 4.5 ± 0.1 × 10⁻³ s⁻¹ (see Table I). The $K_d$ was thus significantly increased from 76 ± 6 to 140 ± 5 nM. These results show that Zn²⁺ potentiates the binding of HRG to Bjκ but inhibits the binding of HRG to Bjλ (see Table I).

Binding of HRG to IgM—The ability of HRG to interact with IgG subclasses (particularly IgG1κ) with high affinity in a Zn²⁺-dependent manner prompted an examination of whether HRG also binds to other Igs such as IgM. For these studies, experiments were conducted to determine whether human b-IgMκ binds to immobilized HRG in an ELISA assay. A concentration-dependent increase in the binding of b-IgMκ to the immobilized HRG was detected. In subsequent studies the IAasys bioensor was used to determine the kinetic constants for the interaction of HRG with immobilized IgMκ. The b-IgMκ (20 ng/cuvette) was immobilized onto the sensing surface of a biosensor cuvette by coupling it to immobilized STP, and the association and dissociation of HRG in PBS-BSA-T buffer was monitored using the biosensor. As shown in Fig. 3A, the binding of HRG to immobilized IgMκ was saturable and was dependent on the concentration of added HRG in the range of 0.5–5 μM. For each concentration of HRG the observed rate constant ($k_{obs}$) was determined from the biosensor data, and the results are presented in Fig. 3B. It was calculated (Table I) that the on-rate and off-rate for the binding of HRG to IgMκ was 0.036 ± 0.005 × 10^5 M⁻¹ s⁻¹ and 7.1 ± 0.1 × 10⁻³ s⁻¹, respectively, and that the $K_d$ for this interaction was 1990 ± 50 nM.

Experiments also were carried out to assess the binding of HRG (1 μM) to immobilized IgMκ in the presence of different concentrations of Zn²⁺ (5–20 μM). The results showed a decrease in the binding of HRG to IgMκ with increasing concentrations of Zn²⁺ (data not shown). Studies of the binding of HRG at different concentrations (0.5–5 μM) to immobilized IgMκ in the presence of 20 μM Zn²⁺ were carried out to determine the effect of Zn²⁺ on the on- and off-rates of the HRG-IgMκ interaction (see Fig. 3B and Table I). These experiments indicated that in the presence of Zn²⁺ the on-rate of the interaction is decreased from 0.036 ± 0.005 × 10^5 to 0.016 ± 0.003 × 10^5 M⁻¹ s⁻¹ (~2.3-fold decrease), whereas the off-rate is increased from 7.1 ± 0.1 × 10⁻³ to 11.8 ± 1.6 × 10⁻³ s⁻¹ (~1.7-fold increase). From the off-rates the $t_{1/2}$ for the interaction of HRG with IgMκ was ~1.6 min and ~1.0 min in the absence and presence of Zn²⁺, respectively; with the analyses employed, these values were not expected to significantly affect measurement of the on-rates. The presence of Zn²⁺ therefore results in a 3.9-fold increase in the $K_d$ for the HRG-IgMκ interaction, increasing it from 1990 ± 50 to 7754 ± 1707 nM (see Fig. 3B and Table I).
show that the affinity for the binding of HRG to IgG1κ is 860-fold greater than the affinity for the binding of HRG to IgG2λ, and the affinity for the binding of HRG to IgG2κ is 820-fold greater than that for binding to IgG2λ (see Fig. 2 and Table I). In contrast, the binding of HRG to IgG3 and IgG4 (when each contained the λ L-chain instead of κ) showed a different pattern. In summary, the results show that although human HRG can interact with all human IgG subclasses, the kinetics of binding are dependent not only on the particular subclass (or constituent H-chain) of the IgG molecules but also on the type of its constituent L-chains. Since HRG has been shown to inhibit the formation of IICs (16), these data provide the first evidence for a functional difference between the role of κ and λ L-chains of immunoglobulin.

Recently we showed that the affinity of the binding of HRG to pooled human IgG was potentiated 4–5-fold by the presence of physiological concentrations (20 μM) of Zn2+ (16). In the present study, an examination of the effect of Zn2+, added in the form of ZnCl2, on the binding of HRG to different IgG subclasses revealed that only the HRG-IgG1κ interaction was significantly affected, with the Kd approximately a 6-fold lower in the presence of Zn2+ (Table I). In contrast to its effect on the HRG-IgG1κ interaction, the presence of Zn2+ had little or no effect on the HRG-IgG1α interaction. In fact, Zn2+ further accentuated the influence of the L-chain isotype on HRG-IgG1 binding, with the Kd of 0.6 nM and 266 nM (a 443-fold difference) for the binding of HRG to IgG1κ and IgG1α, respectively.

Zn2+ is an interesting regulator of the IgG1κ-HRG interaction, and the level of Zn2+ is reported to vary in tissues (28) with relatively large amounts of Zn2+ being released locally by degranulating platelets (28, 29). The question arises, therefore, as to whether the findings on the effects of Zn2+ are physiologically relevant. The concentrations of free Zn2+ in plasma is reported to be 10−9 M, but the real concentration is difficult to determine (30). Evidence suggests that in plasma a large proportion (98%) of the Zn2+ is bound to proteins like albumin (and HRG), and a small proportion presumably exists complexed to other metabolites such as amino acids and citrate, which can bind Zn2+ weakly (26). The binding buffer used in all the studies described in this work contained 1% BSA, and Zn2+ was always equilibrated in the binding buffer before the analysis of protein interactions. As Zn2+ interacts physiologically with the BSA and the molar concentration of BSA was at least 10 times higher than that of the added Zn2+, it could be expected that the free Zn2+ concentration in the binding buffer used in the experiments would be buffered to near physiological levels. Our previous studies (16) showed that the binding of HRG to IgG is decreased significantly (compared with the binding in the absence of any added Zn2+) after adding 1 mM EDTA alone or after adding 20 μM Zn2+ and 1 mM EDTA (16). Similarly, in the present work, an effect of EDTA was observed on the interaction of HRG with the κ L-chain and with IgGκ even in the absence of added Zn2+, and essentially identical binding data could be obtained using HEPES instead of phosphate in the binding buffer (data not shown). These findings indicate that an effect of Zn2+ is already apparent (presumably due to trace amounts of Zn2+ in the buffer or the BSA) in the absence of any added Zn2+ and, hence, that the observed effects of Zn2+ occur at Zn2+ concentrations that are physiologically relevant.

The finding that HRG binds with different affinities to different IgG subclasses depending upon whether κ or λ L-chains are present raised the intriguing possibility that HRG binds directly to the κ or λ L-chains of IgG. An analysis of the binding of HRG to BJ proteins with κ or λ L-chains revealed that HRG binds to both BJκ and BJλ but with different kinetics, resulting in the affinity of the binding of HRG to immobilized BJκ being 82-fold higher than that for the binding to immobilized BJλ (see Table I). Interestingly, the presence of Zn2+ increased the affinity of the binding of HRG to BJκ but decreased that for the binding of HRG to BJλ, resulting in the affinity for the binding of HRG to BJκ being 814-fold higher than that for the binding to BJλ. Consistent with an effect of Zn2+ on the HRG-κ L-chain interaction (see above), these findings suggest that the presence of the κ L-chain in IgG1κ and IgG2κ facilitates the interaction of HRG with these IgG subclasses.

Structure studies have shown that most of the amino acid sequence differences between the different IgG subclasses are located in the hinge region, which may give rise to differences in the length and flexibility of this region of the IgG molecule and, hence, to possible functional differences between the different IgG subclasses. Although the amino acid sequence of the constant region of the κ L-chain is different from that of the constant region of the λ L-chain, the two regions are homologous and structurally related. Less pronounced is the structural homology between the variable regions of the κ and λ L-chains (31). The fact that our data show the binding of HRG to IgG is profoundly influenced by the L-chain isotype, but that the H-chain isotype also modulates the interaction indicates that the constant regions of H- and L-chains, rather than the variable regions, determine HRG binding. This notion is also supported by our observation that HRG interacted similarly in experiments using different myeloma sources of the same IgG subclass and L-chain isotype (data not shown). Furthermore, our earlier experiments indicate that HRG interacts with Fab fragments of IgG (16) but does not interact with the Fc portion of IgG. Evidence that the L-chain forms a key HRG binding site on IgG comes from our observation that HRG interacts with BJ L-chain dimers of either the κ or λ isotype with reasonably good affinity (Kd = 35 or 76 nM, respectively), and that κ L-chains have a much higher affinity for HRG (813-fold) than λ L-chains in the presence of Zn2+ (data not shown). These findings are consistent with each molecule of HRG interacting with both a H-chain (presumably hinge region or CH1 domain) and an L-chain (constant region) of the IgG molecule. Although a precise determination of the stoichiometry of the interaction between HRG and each IgG subclass was considered beyond the scope of the present study, an estimate of the stoichiometry could be made from the maximum binding at equilibrium of the HRG to the various immunoglobulins. Thus, our biosensor data indicate that approximately one HRG molecule can interact with each immobilized dimeric κ and dimeric λ L-chains, and approximately four HRG molecules can interact with each of the IgG subclasses containing the κ light chain. Of course, these estimates are only approximate, as maximum binding levels are affected by dissociation rates and possible inactivation of HRG binding sites on immunoglobulins following their immobilization. Clearly, the mechanism(s) and precise regions of these molecules involved in the interaction of HRG with IgG and individual L-chains remain to be determined and await further studies using techniques like site-directed mutagenesis and x-ray crystallography.

BJ proteins are of pathological importance in diseases associated with elevated levels of L-chains in the blood circulation, where the deposition of BJ proteins can be associated with renal and/or systemic L-chain deposition disease. Approximately 80% of patients with L-chain deposition disease have L-chain deposits (renal tubular casts) due to deposition of κ L-chain (32, 33). Evidence suggests that the insolubilization and aggregation of the unfolded κ L-chain protein may occur via intermolecular hydrophobic interactions (34), but hitherto, no mechanism capable of protecting against the insolubiliza-
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deposition and distribution of these light chains in either the kidney or the blood vessel wall has been reported. The present findings that HRG binds strongly to BJ proteins, particularly BJk, suggests that HRG may be an important factor in regulating the insolubilization, aggregation, and pathological effects of BJ proteins in patients with L-chain deposition disease.

The IgG form of rheumatoid factor is purported to be the more pathogenic form of Ig in relation to rheumatoid arthritis and systemic lupus erythematosus (35), despite the predominant form of rheumatoid factor detected in the sera of patients with rheumatoid arthritis and systemic lupus erythematosus being IgM. It was important, therefore, to determine whether HRG also interacts with IgM. Kinetic studies of the binding of soluble HRG to immobilized IgMx showed that HRG binds to the IgM with an affinity that is ~650-fold lower than for the binding of HRG to IgGlk (see Fig. 3 and Table I). Interestingly, the presence of 20 μM Zn2+ reduced the affinity of the HRG-IgMx interaction by ~3.9-fold. It is noteworthy that in these experiments the binding of HRG was specific for each immobilized ligand, as the interaction of HRG with IgM (and with other immobilized ligands tested in the present work) was inhibitable by preincubation of the HRG with a 4-fold molar excess of the soluble ligand (not shown). These results show that HRG binds to IgMx with relatively low binding affinity (Kd ≥ 2 μM for IgMx). In light of the concentration of HRG in normal plasma of ~2 μM, however, it is unlikely that the binding of HRG to the IgM is of physiological relevance. The results nonetheless further highlight the strong modulating influence of the H-chain isotype on the binding of HRG to immunoglobulins.

A key feature of IC formation is that, following the initial antigen-dependent cross-linking of antigen and antibody, there is an antigen-independent phase of IC insolubilization (36). Until recently, only the plasma proteins Clq and C3b have been implicated in modulating the formation of IC in vitro (37). We have since discovered that HRG or the 30-kDa N-terminal fragment of HRG can markedly inhibit the formation of IC between ovalbumin and polyclonal rabbit anti-ovalbumin IgG (16). Furthermore, we showed that normal human plasma is an effective inhibitor of IIC formation but that the specific depletion of HRG from human plasma results in a plasma preparation that dramatically enhances IIC formation (16). These findings imply that HRG is the dominant endogenous inhibitor of IIC formation in normal plasma. Consistent with this conclusion we showed that HRG also has a high affinity binding site for Clq, with the IgG and Clq binding sites both being localized to the amino-terminal 30-kDa domain of human HRG (16). As well as inhibiting IIC formation, HRG appears to play a role in the clearance of ICs from the blood circulation. Thus, our recent studies show that the incorporation of HRG into ICs facilitates the binding of these ICs to monocytes (38), which can clear the ICs by phagocytosis. In addition, it is well known that the binding of ICs to erythrocytes promotes the clearance of ICs from the blood circulation by the reticuloendothelial system (39). Since HRG is known to bind to erythrocytes via its heparin/heparan sulfate binding domain (12, 23), our finding that HRG binds IgG also suggests that HRG may enhance the binding of ICs to erythrocytes. Coupled with the finding that HRG inhibits the formation of ICs, therefore, the present study strongly suggests that the L-chain isotype of IgG molecules determines the size, clearance, and tissue distribution of IgG containing IC in vivo. This scenario is particularly likely since IgGlk and IgG2 generally constitute 90% of the IgG molecules in normal human plasma (22).

Another important implication of the present work follows from the fact that both soluble and insoluble ICs can be generated during an immune response to an antigen and that ICs are likely to be important initiators of localized inflammatory reactions to pathogens (17, 19). The differential binding of HRG to IgG subclasses containing κ and λ L-chains suggests that in the presence of HRG some ICs may remain in a soluble form and thus be cleared faster than others (e.g. IgGlk-containing ICs may be cleared faster than IgGλ-containing ICs). Furthermore, it is conceivable that ICs that are not cleared by the reticuloendothelial system (19, 29) may serve as long-lasting depots of antigen for the maintenance of immunological memory. The ability of IgG molecules to interact with HRG and consequently form soluble versus insoluble ICs depending on the L-chain isotype they express therefore also suggests functional differences in the role of the κ and λ L-chains in regulating this process.

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