IgG RHEUMATOID FACTORS AND STAPHYLOCOCCAL PROTEIN A BIND TO A COMMON MOLECULAR SITE ON IgG

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The etiology of rheumatoid arthritis remains unknown. Increasing evidence, however, indicates that both cellular and humoral immunity are involved in maintaining the chronicity of inflammation in this disease (1). The involvement of humoral immunity is largely through the formation of immune complexes. The predominant immune complexes contain rheumatoid factors (RF),[1] which are antibodies directed to antigenic determinants on the Fc region of IgG. These antibodies may belong to any class of immunoglobulins, and are termed IgG rheumatoid factors (IgG-RF), IgM rheumatoid factors (IgM-RF), etc. Self-associating IgG-RF form unique immune complexes in that these IgG molecules serve as both antigens and antibodies. These molecules form the intermediate complexes in the sera (2), and have been isolated from the synovial fluids (F. Nardella, D. C. Teller, and M. Mannik, unpublished results) of patients with rheumatoid arthritis. IgG-RF are the most prevalent antibodies synthesized by plasma cells in the human rheumatoid synovium (3–5). Furthermore, the IgG-RF can drive inflammation by activation of complement (6, 7), and by interaction with human monocytes (8). Why rheumatoid factors arise in patients with rheumatoid arthritis and in other diseases is unknown.

Previous studies (reviewed in 9) have shown that the antigenic specificities of IgM-RF are directed to either nongenetic or genetic antigenic determinants. Some of these determinants were identified on the Cγ3 domain, and others were inferred to be on the Cγ2 domain. The Ga nongenetic antigenic determinant was most prevalent, and was present on all IgG subclasses except IgG3 (10). Previous studies from this laboratory (11) have shown that identical antigenic determinants for IgG-RF were present on the Fc fragments of normal IgG and IgG-RF, and were absent from the Cγ3 domain. Knowledge of the antigenic determinants for IgG-RF in molecular detail might provide a clearer picture of the mechanism of self-association of these molecules, and provide clues to the initiation of RF production.

We now have determined the site on the IgG molecule that is involved in the

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[1] *Abbreviations used in this paper:* KD_{50}, concentration of inhibitor at 50% of control binding; RF, rheumatoid factor, SPA, staphylococcal protein A.

self-association of IgG-RF, and have shown that it is in the same location and involves some of the same amino acid side chains as the site that binds staphylococcal protein A (SPA). SPA is found in the cell wall of most strains of *Staphylococcus aureus.* It has a molecular mass of 42 kilodaltons (kD), and has four highly homologous regions, each able to bind to the Fc regions of the IgG from various species. In man, SPA binds to the Fc regions of human subclasses IgG1, -2, and -4, but not IgG3 (reviewed in 12). The complex of an SPA monovalent subunit, fragment B, with human Fc has been crystallized, and the amino acid side chains involved in the binding determined by x-ray crystallography (13, 14). The coincidence of binding sites for IgG-RF and SPA suggests new approaches for study of the etiology of rheumatoid arthritis.

Materials and Methods

Affinity Isolation of IgG-RF. IgG-RF was isolated from the plasma of patients JJ and EG by affinity chromatography on human monomeric IgG-agarose columns. The human monomeric IgG columns were prepared as described previously (11). The columns were equilibrated at 4°C in pH 8.8 borate buffer (0.2 M borate, 0.15 M NaCl) to minimize the nonspecific adsorption of non-RF IgG (15). This pH also, as shown below, results in maximum interaction energy of the RF-antigen bond. 1–3 ml of JJ or EG plasma were applied to the affinity columns equilibrated in the starting buffer. RF were eluted with acetate buffer (0.1 M acetate, pH 3.5) after washing the columns with ~400 ml of starting buffer. The columns were cycled through the starting and eluting buffers until the absorbance at 280 nm of the acid cycle was <0.003. The pH 3.5 acetate eluates were pooled and concentrated using stirred, positive pressure concentrators with Diaflow YM 10 membranes (Amicon Corporation, Lexington, MA). The IgG-RF from patient JJ were further purified by gel filtration on a Sepharose S-300 column (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated in phosphate buffer (0.05 M phosphate, 0.15 M NaCl, pH 7.3). The affinity isolates from patient EG were further purified by gel filtration on an S-300 column equilibrated in 0.1 M acetate buffer to remove the IgM-RF. The IgG peak was pooled, concentrated, and applied to an S-300 column equilibrated in phosphate buffer. The resultant IgG-RF were pooled and concentrated as described above and, contained <5% IgM and IgA, as determined by radial immunodiffusion (Immunoplate III; Travenol Laboratories, Inc., Costa Mesa, CA). $F(ab')_2$ fragments were prepared from the isolated IgG-RF by pepsin digestion and subsequent Sepharose S-300 gel filtration with the column equilibrated in acetate buffer. JJ Fab fragments were prepared from the isolated complexes as described previously (11).

Preparation of Human IgG and IgG Fragments. Human IgG was prepared from Cohn fraction II (Miles Laboratories, Inc., Elkhart, IN) by DEAE-Sephacel (Pharmacia Fine Chemicals) ion-exchange chromatography in 0.1 M Tris buffer, pH 8.6. Human $F(ab')_2$ and pFc' fragments were prepared and isolated from human IgG, and human Fc was prepared and isolated from papain-digested IgG, both as described previously (11). Rabbit IgG was prepared from rabbit Cohn fraction II (Miles Laboratories, Inc.) by gel filtration on an S-300 column equilibrated in borate buffer. Rabbit $F(ab')_2$ and pFc' fragments were prepared from Cohn fraction II by pepsin digestion (16) and S-300 gel filtration. Rabbit Fc fragments were prepared and isolated from rabbit Cohn fraction II by the method of Porter (17). Rabbit Facb fragments were prepared and isolated from Cohn fraction II by a modification of the method of Connell and Porter (18). 380 mg of rabbit Cohn fraction II was dissolved in 14 ml of 0.04 M acetate buffer at pH 5.5. The pH was lowered to 2.5 with 1 M HCl and allowed to incubate for 15 minutes at 37°C. The pH was raised to 7.3 with 1 M NaOH and 4.32 mg of porcine plasmin (Sigma Chemical Co., St. Louis, MO) was added. After incubation for 30 min at 37°C, the digestion mixture was immediately gel filtered on a G-200 column (Pharmacia Fine Chemicals) in 0.05 M phosphate buffer at 4°C. The initial peak was pooled, concentrated to 22 ml using a stirred, positive pressure concentrator with YM 10 membranes, dialyzed into 0.01 M

sodium acetate, pH 5.5, and applied to a CM 52 cation-exchange column (Whatman Chemicals, Maidstone, Kent, United Kingdom) in the same buffer. The Facb-containing peak was the second peak to emerge in a 0.01–0.1 M sodium acetate, pH 5.5, linear gradient. This peak was pooled and concentrated, and applied to CL 4 B SPA column (Pharmacia Fine Chemicals) to remove any remaining intact IgG. Human IgG3 McD and EV were prepared from plasma by starch block electrophoresis and S-300 gel filtration of the monoclonal peaks.

Staphylococcal Protein A. SPA was obtained from Pharmacia Fine Chemicals, and from IMRE Corp. (Seattle, WA). Fragment D of SPA was obtained from J. Sjöquist (University of Uppsala, Sweden).

Chemical Modifications of IgG and IgG RF. Chemical modification of histidines in IgG and IgG-RF was done with diethylpyrocarbonate (19). The modifications were followed spectrophotometrically, and reversed with hydroxylamine. Modification of lysines was done by reductive methylation at pH 9 using formaldehyde as the carbonyl donor and sodium borohydride as the reductant (20). The degree of amino group substitution was determined by trinitrobenzene sulfonic acid assay (21). Modification of tyrosines was done with tetranitromethane (22) and *N*-acetylimidazole (23). The degree of modifications was determined spectrophotometrically. The *N*-acetylimidazole modifications were reversed with hydroxylamine.

pH Titration Studies. A pH titration for the interaction of Fab fragments of JJ IgG-RF and normal IgG was done by high-speed sedimentation equilibrium ultracentrifugation. The ultracentrifugation techniques have been described previously (11). The Fab RF fragments and normal human IgG, at a molar ratio of 2:1, were dialyzed against 0.05 M phosphate buffer at the appropriate pH. Phosphate buffers were kept at a constant ionic strength of 0.28 OsM by varying the concentration of NaCl. The buffers were rendered 0.005 M in acetate between pH 4.35 and 4.85, and 0.005 M in ethanolamine between pH 8.85 and 10.35, to maintain buffering capacities. Three samples with initial protein concentrations of 1.0, 0.7, and 0.4 mg/ml were centrifuged at 15,000 rpm for 24 h at 20°C at each of the pH values tested between pH 4.35 and 11.35. The partial specific volumes for both components were assumed to be 0.738 ml/g (24). Using a molecular mass of 47.1 kD for JJ Fab, and 141.7 kD for IgG, as determined by analytical ultracentrifugation, the data were analyzed for complex formation by the method of Benhke et al. (25) to obtain equilibrium constants for the 1:1 complex formed by the two reactants. It was previously shown (11) that the antigenic valence for normal IgG in this interaction is one. The equilibrium constant at each pH is K_c = [Fab-IgG]/[Fab][IgG]. For the linked function analysis, the reaction is considered to be reactants $\leftrightarrows$ products. The data were fitted as $\log K_c = cst + \sum_{\text{products}} n_p \log (K_p + [H^+]) - \sum_{\text{reactants}} n_r \log (K_r + [H^+])$, where *cst* is the value of $\log K_c$ at high pH. K_p and K_r are proton dissociation constants. pH titrations were also done for interactions of intact radiolabeled JJ IgG-RF and EG IgG-RF, with normal IgG using a solid-phase equilibrium radioimmunoassay described below. The percent binding of 7×10^{-9} M radiolabeled JJ IgG-RF or 7×10^{-10} M EG IgG-RF to aggregated IgG bound to the test tubes, expressed as 7×10^{-8} M IgG monomers, was determined at the various pH in the same buffers used for the analytical ultracentrifugation experiments. The percent binding of radiolabeled JJ RF or EG RF were corrected for nonspecific binding by subtraction of binding of normal, radiolabeled IgG to the IgG coated tubes. Under these circumstances, with low percent binding of RF, the value of $\log K_c$, the association constant for complex formation, should be proportional to the log of the percent binding, to a first approximation. We found that $\log K_c = S \log P$ fitted the data most smoothly, where P is the corrected percent binding of IgG RF to the test tubes, and S is the scale factor, determined as a maximum likelihood estimate parameter together with the equilibrium constant parameters.

Solid-phase Equilibrium Radioimmunoassay. For the inhibition radioimmunoassays, 101–193 ng of ^{125}I–JJ IgG-RF, or 51–110 ng of ^{125}I–EG IgG-RF (previously determined to result in maximum percent bindings) in a reaction volume of 100 μl were mixed with increasing concentrations of inhibitors in borate buffer, with 0.1% bovine serum albumin, and added to polypropylene tubes previously coated for 2.5 h at room temperature with

2 mg/ml heat-aggregated human IgG. Heat-aggregated human IgG was prepared by heating IgG obtained as described above for 15 min at 63°C. The remainder of the adsorptive sites were blocked with 0.1% bovine serum albumin in borate buffer for 90 min before adding the mixtures of IgG-RF, with or without inhibitors. The IgG-coated tubes with the mixtures were stored overnight at 4°C, after which the top (T) half were removed from the bottom (B), both halves counted in an automatic well-type gamma counter, and the percent binding determined as $100 \times (B - T)/(B + T)$. The assays were performed in triplicate, converted to 100% on the ordinate for ease of interassay comparisons, and expressed as mean ± SEM. The observed data were fit to the binding equation $P = [(KD_{50} \times P_{min}) + (I \times P_{max})]/(KD_{50} + I)$, where P is the percent binding, P_{min} is the asymptote at low I, P_{max} is the asymptote at high I, KD_{50} represents the dissociation constant, and is equal to I when P is 50% of control binding; I is the concentration of inhibitor. The fitting was accomplished with a computer program using a weighted nonlinear least squares function. For weak inhibitors, KD_{50} expressed in the tables are extrapolated values, when a computer fit could be obtained. For direct-binding assays, the binding of the radiolabeled IgG RF to the solid-phase IgG were studied over a range of 100–1,000 ng. The other details of the assay are as described for the inhibition assays.

Computer Drawing of Stereo Pair Diagram of ½Fc. The stereo pair diagram of ½Fc, showing the amino acid side chains involved in the binding of SPA, was drawn on an AED 767 high-resolution color rastergraphics terminal using a VAX 11/780 computer and the MOMUS program. The program MOMUS (Multiport molecular structure drawing and comparison program) was written by K. Watenpaugh of the Department of Biological Structure, University of Washington.

Results

Localization of Antigenic Determinants to IgG Domains. We used a solid-phase equilibrium radioimmunoassay to examine the interaction of IgG-RF with antigens. In this assay, purified, radiolabeled IgG-RF or $F(ab')_2$ fragments of IgG-RF were allowed to bind to solid-phase human IgG. The inhibition of this binding was examined with a variety of preparations. The degree of inhibition reflects the presence of appropriate antigenic determinants on the test substance. The binding of IgG-RF could be readily inhibited by intact human or rabbit IgG, as well as by Fc fragments of these proteins, although a difference was observed between the two IgG-RF with rabbit IgG and Fc. In contrast, $F(ab')_2$ fragments, Facb fragments (this fragment of rabbit IgG contains all domains except $C\gamma3$ [14]) and pFc′ fragments ($C\gamma3$ domains of IgG) were poor inhibitors (Table I and Fig. 1). Thus, the RF did not interact with the $C\gamma2$ or $C\gamma3$ domains alone, but required the presence of both domains. It had already been shown (11) that normal Fc fragments and Fc fragments from IgG-RF reacted identically with the $F(ab')_2$ fragments of IgG-RF by sedimentation equilibrium ultracentrifugation, demonstrating that unique antigenic determinants did not exist on the Fc fragments of IgG-RF.

A previously recognized antigenic determinant for IgM-RF is present (10) on subclasses IgG1, -2, and -4, and is absent from IgG3. The two IgG3 myeloma proteins tested in these studies were ineffective inhibitors of the binding of the IgG-RF to solid-phase IgG (Table I and Fig. 1). This finding suggested that the antigenic determinant for self-association of IgG-RF may be comparable to the so-called Ga site for IgM-RF.

As already noted, SPA also binds human IgG1, -2, and -4, and not IgG3 (reviewed in 12). SPA and its active monovalent subunit, fragment D (7 kD) readily inhibited the binding of the $F(ab')_2$ fragments of both IgG-RF to solid-

TABLE I
Inhibition of Binding of ^{125}I–JJ and –EG IgG-RF to Solid-phase IgG by Human and Rabbit IgG, IgG Fragments, and SPA

Inhibitor	KD_{50} (±SE)	
	JJ	EG
	μM	
Human IgG	1.45 ± 0.49	0.60 ± 0.21
Human Fc	2.43 ± 0.14	1.34 ± 0.25
Human $F(ab')_2$	103 ± 140*	74.0 ± 275*
Human pFc'	116 ± 33.2*	86.1 ± 17.7*
Rabbit IgG	4.06 ± 1.13*	0.29 ± 0.07
Rabbit Fc	7.36 ± 1.80*	1.04 ± 0.49
Rabbit $F(ab')_2$	30.01 ± 3.34*	15.18 ± 8.42*
Rabbit pFc'	No inhibition	37.40 ± 11.52*
Rabbit Facb	12.76 ± 1.97*	17.98 ± 8.25*
Human IgG3 McD	5.03 ± 1.81*	8.22 ± 2.73*
Human IgG3 EV	6.61 ± 1.90*	4.95 ± 1.22*
SPA	0.02 ± 0.003	0.01 ± 0.004
Fragment D of SPA	0.05 ± 0.01	0.15 ± 0.04

The following molecular masses (kD) were used in calculation of molar concentrations of human and rabbit IgG and IgG fragments: IgG, 150.0; Facb, 125.0; $F(ab')_2$, 100.0; Fc, 50.0; and pFc', 25.0. A small KD_{50} indicates a good inhibitor, and conversely a large KD_{50} indicates a poor inhibitor.

* Extrapolated KD_{50}. See legend to Fig. 1.

phase IgG by binding to the IgG and thus preventing binding of the RF preparations (Table I and Fig. 1).

Collectively, these experiments indicated that the antigenic determinant for self-association of IgG-RF required intact Cγ2 and Cγ3 domains, may be comparable to the Ga antigenic determinant described for IgM-RF, and may involve the same region that binds SPA.

pH Titration Studies. To obtain information regarding the specific amino acid residues involved in the IgG-RF-antigen interactions, a pH titration for the interaction of Fab fragments of JJ IgG-RF and normal IgG was done by high-speed sedimentation equilibrium ultracentrifugation. pH titrations were also done for the interactions of intact, radiolabeled JJ and EG IgG-RF with normal IgG using the solid-phase equilibrium radioimmunoassay. If an ionizable amino acid side chain was involved in the RF-antigen interactions, then protonation or deprotonation would perturb them at a pK characteristic of the involved group. The same plateaus and inflections were present in the plots obtained by radioimmunoassays as in the ultracentrifuge study, indicating that the processes occurring in the radioimmunoassay were the same as those measured by sedimentation equilibrium ultracentrifugation. Changes in the binding occurred with apparent pK (pK_{app}) of 5.0, 7.0, and 10.4 (Fig. 2, *A* and *B*) indicating the involvement of Asp and Glu, His, and Lys or Tyr respectively for both IgG-RF. The carboxylate involvements were not pursued further, since low pH dissociates RF-antigen

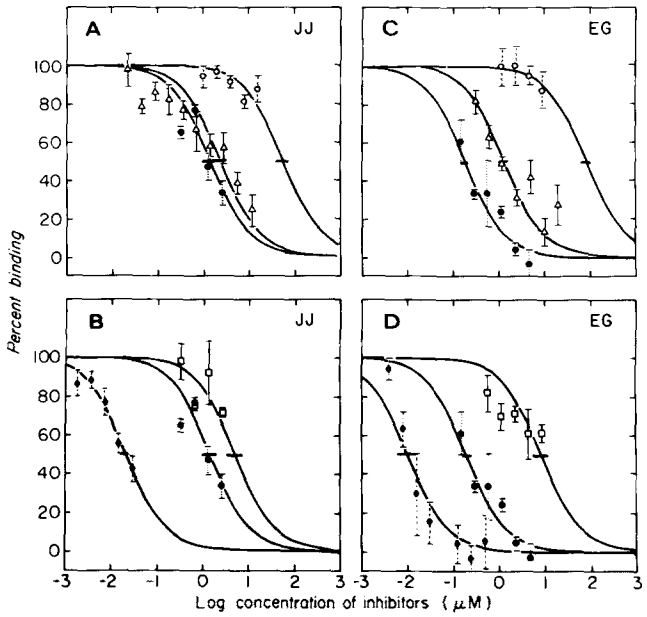

FIGURE 1. Inhibition of binding of ^{125}I–JJ (*A* and *B*) and –EG (*C* and *D*) IgG-RF or F(ab')$_2$–RF to solid-phase IgG by human IgG (●), human Fc (△), human pFc' (○), human IgG3 McD (□), and SPA (◆). The $KD_{50} \pm SE$ are represented as the horizontal bars at the midpoints of the fitted curves. For weak inhibitors, the KD_{50} expressed in the tables are extrapolated values, estimated when a computer fit could be obtained.

interactions and induces conformational changes that are difficult to distinguish from discrete carboxyl group involvement. The putative lysyl residues were eliminated by chemical modification studies as shown below. Thus, histidyl and tyrosyl residues are the important determinants of the neutral and alkaline areas of the pH titration. The number of groups involved could not be determined from the data. For the purposes of curve fitting, two carboxylate, three histidyl, and two tyrosyl residues were chosen for the linked-function model represented in Fig. 2*C*. Independent of the model used, ionization of His decreases complex formation, as does ionization of tyrosine. The direction of the pK shifts implies that His and Tyr are buried in complex formation.

Chemical Modification Studies. The pH titration studies of the IgG-RF and IgG interactions described above indicated that amino acid side chains of histidines and lysines and/or tyrosines were involved. To determine whether the histidine residues involved were on the antigen or antibody sides of the interactions, chemical modification of IgG and the IgG-RF was done with diethylpyrocarbonate. The modifications were followed spectrophotometrically, and reversed with hydroxylamine (19). The preparations treated with diethylpyrocarbonate showed no evidence of tyrosine modification, as determined by the absence of changes in optical absorbance at 278 nm.

With increased modification of histidines on IgG, its ability to inhibit the binding of IgG-RF was decreased. Furthermore, when the lower degrees of modification were reversed with hydroxylamine, the inhibitory activities of IgG were recovered (Table II). The reversal of heavily modified IgG could not be

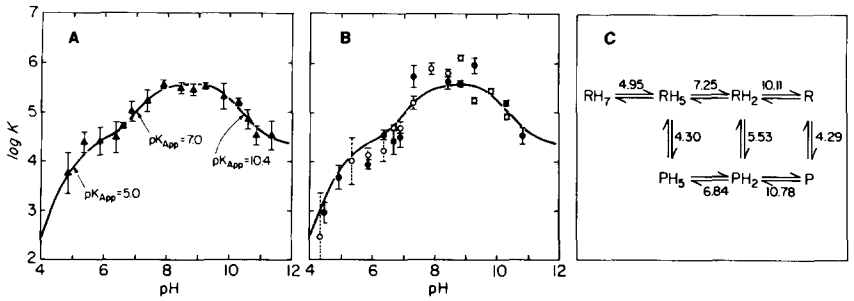

FIGURE 2. pH dependence of the interaction of RF with normal IgG. (*A*) Equilibrium constants determined by high-speed sedimentation equilibrium ultracentrifugation for the interaction of Fab fragments of JJ IgG-RF and normal human IgG, at a molar ratio of 2:1 in 0.05 M phosphate buffer at the appropriate pH. Three samples with initial protein concentrations of 1.0, 0.7, and 0.4 mg/ml were centrifuged at 15,000 rpm for 24 h at 20°C at each pH. The line drawn in the figures is for the model in *C*. Independent of the numbers of groups on the model, the midpoint pH of the decrease in the alkaline range is 10.4, corresponding to lysyl or tyrosyl residue(s). The midpoint pK in the neutral range is 7.0, and corresponds to histidyl residue(s); the drop in log *K* at the very lowest pH values corresponds to carboxylate (Asp or Glu) residues. (*B*) pH dependence of RF binding by radioimmunoassay. The percent binding of radiolabeled JJ RF (○) (7×10^{-9} M) or EG RF (●) (7×10^{-10} M) at various pH were corrected for nonspecific binding by subtraction of binding of normal, radiolabeled IgG to the IgG-coated tubes. The molar amount of aggregated IgG bound to the test tubes as antigen was 7×10^{-8} M, expressed as IgG monomers. Both IgG-RF behaved identically in this analysis. The line drawn in the figure is for the same model and parameters as in *A*. (*C*) Model used for fitting the data in *A* and *B*. Numbers associated with the horizontal equilibria are pK_a values; numbers adjacent to the vertical arrows are log *K* values for these equilibria. In this particular model, two carboxylate, three histidyl, and two tyrosyl residues were assumed, as denoted by the subscripts of *H* for the reactants (*R*) and products (*P*). Other models with different numbers of dissociable protons fit the data almost as well as the model shown.

achieved, presumably because of formation of dimodified histidyl residues. In contrast, the modifications of histidines on IgG-RF caused no or only minimal decrease in binding of these molecules to the antigen (Table III). These data indicate that the histidines involved in these antigen-antibody interactions are on the antigen molecules. Six histidine residues exist on the Fc portion of each γ chain of IgG1 EU (26). Two of these (His 268 and His 285) are not in the Cγ2-Cγ3 interface region (27), and are unlikely to be involved. Histidine 429 is inaccessible to solvent. Thus, one or more of the three His residues in the Cγ2-Cγ3 interface (310, 433, and 435) are involved (Fig. 3*A*). These same histidine residues are involved in the binding of SPA to human IgG (13, 14) (Fig. 3, *B* and *C*). Molecules of the IgG3 subclass do not bind SPA because of the substitutions of arginine and phenylalanine for histidine 435 and tyrosine 436 (13, 14). IgG3 poorly inhibited the binding of both IgG-RF, as noted above, indicating that His 435, and one or both of His 433 and 310 are involved in the binding.

Reductive methylation of IgG and the IgG-RF was done to determine if lysines were involved, and, if so, whether they were stationed on the antigen or antibody sides of the interactions. Reductive methylation was accomplished at pH 9 using formaldehyde as the carbonyl donor and sodium borohydride as the reductant (20). Under these conditions, stable dimethylamino groups are formed and other side chain groups do not give stable derivatives. As assessed by ultraviolet

TABLE II

Inhibition of Binding of ^{125}I–JJ and –EG IgG-RF to Solid-phase IgG by Chemically Modified Human IgG

Modification	JJ		EG	
	Degree of modification	KD_{50} (±SE)	Degree of modification	KD_{50} (±SE)
	%	μM	%	μM
Unmodified IgG	—	1.45 ± 0.49	—	0.60 ± 0.21
Diethylpyrocarbonate (histidines)	26	4.07 ± 1.16*	30	4.53 ± 1.04*
	90	36.8 ± 23.6*	59	91.1 ± 239*
Hydroxylamine reversed‡		1.21 ± 0.30		1.30 ± 0.29
Reductive methylation (lysines)	46	4.65 ± 1.10	46	1.84 ± 0.38
	81	1.81 ± 0.34	81	0.98 ± 0.12
Tetranitromethane (phenolic ring of tyrosines)	30	3.71 ± 1.82		
	48	No inhibition	48	4.60 ± 1.46*
N-Acetylimidazole (phenolic hydroxyl group of tyrosines)	11	19.8 ± 11.0*	14	4.78 ± 1.92*
Hydroxylamine reversed		3.71 ± 1.89		0.51 ± 0.17

* Extrapolated KD_{50}. See legend to Fig. 1 and Table I.

‡ Refers to 26 and 30% modified IgG for inhibition of JJ and EG IgG-RF, respectively.

spectroscopy, tyrosines were not modified. The degree of amino group substitutions was determined by the trinitrobenzenesulfonic acid assay (21). Modifications of up to 81% of the lysines on IgG caused no decrease in the ability of IgG to inhibit the binding of IgG-RF to the solid-phase antigens (Table II). Modifications of up to 86% of the lysines on JJ IgG-RF, and 79% of the lysines on EG IgG-RF reduced their ability to bind by 21% and 51%, respectively (Table III). Thus, lysines are not involved on the antigen sides of the interactions. It is likely they are not directly involved on the antibody sides of the interactions either, but rather, extensive modification may have caused conformational changes resulting in a partial decrease of antibody binding.

To explore the role of tyrosines in the bindings, two reagents, tetranitromethane and *N*-acetylimidazole, were used for chemical modifications. Under mild conditions, tetranitromethane causes nitration of the phenolic ring of tyrosine, which can be followed spectrophotometrically (22). Modification of up to 48% of tyrosines caused a significant decrease in the ability of IgG to inhibit the binding of the IgG-RF to solid-phase IgG (Table II). Likewise, modification of 57% of the tyrosines on JJ IgG-RF reduced its binding by 52% (Table III). *N*-acetylimidazole reacts preferentially with tyrosyl residues by acetylating phenolic hydroxyl groups. This modification is also reversible with hydroxylamine (23). Modification of 11% and 14% of the tyrosines on IgG preparations decreased their ability to inhibit JJ and EG IgG-RF binding to solid phase IgG. Restoration of inhibition to near normal levels occurred after treatment with hydroxylamine (Table II). Modification of tyrosines on IgG-RF markedly reduced their ability

TABLE III
Binding of Chemically Modified ^{125}I–JJ and –EG IgG-RF to Solid-phase IgG

Modification	JJ		EG	
	Degree of modification	Binding (percent of control ± SE)*	Degree of modification	Binding (percent of control ± SE)*
	%		%	
Unmodified RF	—	100 ± 21	—	100 ± 8
Diethylpyrocarbonate (histidines)	29	125 ± 23	50	82 ± 9
	58	101 ± 22	89	65 ± 5
Reductive methylation (lysines)	43	79 ± 2	42	68 ± 10
	86	90 ± 3	79	49 ± 10
Tetranitromethane (phenolic ring of tyrosines)	14	70 ± 6		
	57	48 ± 8		
N-Acetylimidazole (phenolic hydroxyl groups of tyrosines)	6	2 ± 9	ND	12 ± 5
Hydroxylamine reversed		43 ± 8	ND	57 ± 7

* Maximum percent binding of unmodified IgG RF is designated as 100%. The maximum binding of the IgG RF after chemical modifications is relative to unmodified IgG RF. The standard errors of percent bindings were calculated from the data by a formula for propagation of error (35). ND, not determined.

to bind to solid phase antigen. Treatment with hydroxylamine partially restored this binding (Table III). These studies indicated involvement of Tyr on the antigen and on the antibody sides of the RF-antigen interactions. Of the nine Tyr on the Fc portion of each γ chain of IgG1 EU, only Tyr 436 is in the Cγ2-Cγ3 interface region (27) (Fig. 3*A*). This residue is also involved in binding SPA (13, 14) (Fig. 3, *B* and *C*).

Discussion

We have shown that amino acids His 435, Tyr 436, and one or both of His 310 and His 433 are involved in the antigenic determinant for self-associating IgG-RF. The accessible atoms of these residues form an ellipse with semiaxes 9.6 and 13.0 Å that describes the size of this region of the Fc fragment. This is only slightly larger than the cylindrical radius of 8.4 Å described by Novotny et al. (28) for antigen-binding sites of antibodies. By the same analysis, the region that binds SPA is larger, with semiaxes of 12.1 and 16.6 Å. These considerations indicate that the antigenic site for RF occupy a subarea of the larger site that binds SPA.

Previous studies from these laboratories (2) indicated a self-association of the IgG-RF from patients with rheumatoid arthritis. Dimers of self-associated IgG-RF were observed that underwent concentration-dependent polymerization. A model was proposed that accounted for the stability of dimers by the formation

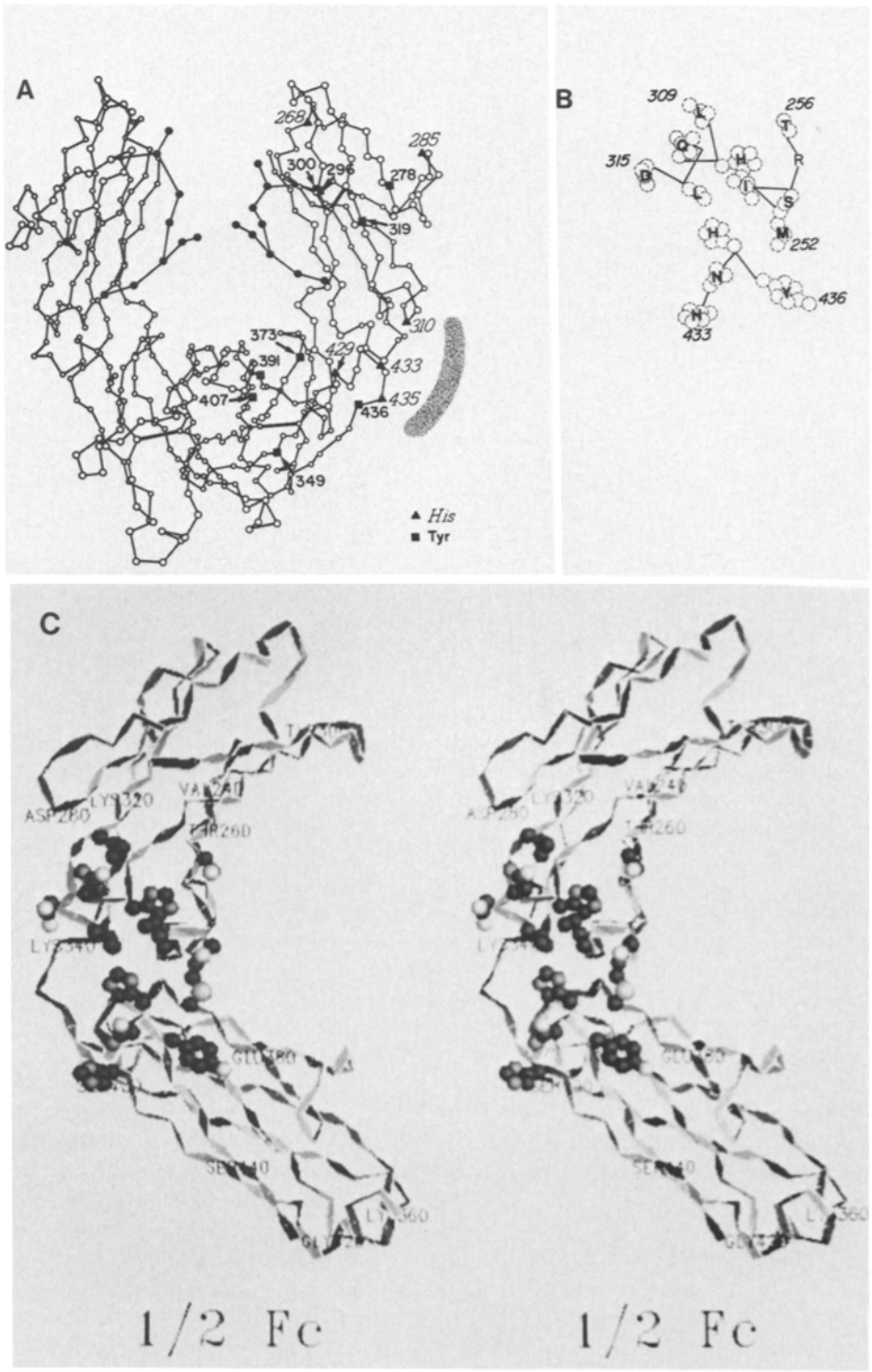

FIGURE 3. Drawings of the Fc regions and details of the binding site for SPA and IgG-RF. (*A*) α-carbon positions (○), and centers of the hexose units (●) of the Fc fragment of human IgG, redrawn with permission from Huber et al. (27). Histidyl residues are denoted by ▲ and tyrosyl residues by ■. The region of the SPA site is designated by the stippled area. (*B*) Schematic orientation of the residues shown in the stereo diagram in *C*. The residues are drawn from the β-carbon, and the backbone is given by a solid line between carbon atoms. The single-letter code is used for residue names. (*C*) Stereo pair diagram of the ½Fc fragment shown in *A*. For clarity, the molecule has been rotated relative to its position in *A*. Every 20th residue is numbered. The atomic shading is (from light to dark) oxygen, sulfur, nitrogen, carbon. The amino acid side chain residues shown are shielded by >20 Å^2 when fragment B of SPA is contacted (14).

of two antigen-antibody bonds between two IgG-RF molecules. The polymerization of dimers to higher molecular forms, however, proceeded with an association constant of $\sim 10^5$/M, which is that expected for one RF-antigen bond. Three observations must be considered in proposing a molecular model for self-association of IgG-RF. First, as just explained, the cyclic dimer is the basic unit for further polymerization (2). Second, previous studies (11) and current observations showed that normal IgG was functionally monovalent for interaction with Fab fragments of IgG-RF. On the other hand, the Fc fragments of normal IgG and of IgG-RF were bivalent, suggesting that when a Fab region of IgG-RF bound to IgG, the Fab arms of IgG were shifted, and sterically blocked the second antigenic site. In the self-association of IgG-RF, however, polymers higher than dimers were formed (2). Therefore, the second antigenic determinant must be available in some of the self-associated IgG-RF molecules. Third, the location of the antigenic site on the Fc fragment, as determined herein, makes an angle of 53° relative to the axis between the amino-terminal domains in the Fab arms of the model of IgG Dob (29). To form the cyclic dimer of two IgG-RF molecules proposed earlier (2), molecular motion of the Fab arms and a resultant strain are necessary. The subsequent molecular distortion most likely dissipates some of the bonding energy, and may induce the exposure of the second antigenic site to allow further polymer formation. Furthermore, the resultant conformation may allow only one RF-antigen interaction in the polymerization of dimers.

The currently available data do not provide sufficient detail to construct a complete molecular model for self-association of IgG-RF or to account for the monovalence of IgG for interaction with IgG-RF. The rotation and contraction of the hinge region proposed by Huber et al. (27) appears to expose the antigenic sites we have identified. This would explain the bivalence of IgG-RF in self-association, but does not address the univalence of IgG for interaction with IgG-RF. Another possible explanation of the monovalency of IgG is that the configuration of the Fc fragment changes upon combination with antibody in the $C\gamma 2$-$C\gamma 3$ region. Since the binding free energy is only 7 kcal/mole or less for one RF-antigen bond formation, and since major changes in conformation of the Fc were not observed in the higher energy binding of the fragment B of SPA in the crystallographic work (13), this possibility seems remote. Finally, we have constructed semiflexible styrofoam models using the coordinate data of IgG Dob (29). In these models, the binding of an Fab fragment of IgG-RF to the identified antigenic site could displace the Fab arms, depending on the angle between the two ligands, and thus account for the observed monovalence of normal IgG, as originally proposed (11).

Abundant evidence indicates that RF play an important role in rheumatoid arthritis. Chronic antigenic stimulation of humans with various infectious agents, including bacteria, has been associated with the development of RF (30). The cause of RF production in rheumatoid arthritis, however, remains unknown. We describe here studies that indicate that the binding site for some IgG-RF is in the same location on the Fc region of IgG, and involves some of the same amino acid side chains as the binding of a bacterial cell wall protein, SPA. Furthermore, this site is likely to be the same as the Ga determinant, described for IgM-RF, since that site was also present on IgG1, -2, and -4, and not on IgG3 molecules,

and was absent from the Cγ3 domain (pFc′ fragments) (31). Although much is known about the biochemistry of SPA binding to IgG, comparatively little is understood about the role of SPA in staphylococcal infections. Cell surface proteins of bacteria are important in the immune response of the host and to the virulence factors of bacteria. The studies presented here suggest a biological relationship between bacterial cell surface proteins that bind to IgG and the production of RF. We speculate that Fc-binding proteins of other infectious agents may bind to this same location. Based on the internal image concept of Jerne (32), others (33, 34) have speculated that autoantibodies arise as autoanti-idiotypes to antibodies against certain pathogens. The relationship between RF and SPA described here raises the testable hypothesis that RF arise as autoanti-idiotypes to antibodies directed against Fc-binding proteins of infectious agents. If such mechanisms exist, the autoantiidiotypic responses may well be under genetic control, which may explain the association of rheumatoid arthritis with certain histocompatibility antigens.

Summary

The antigenic determinant on the Fc region of human IgG for two IgG rheumatoid factors (IgG-RF) from patients with rheumatoid arthritis were investigated in detail. The RF did not interact with IgG fragments that contained the Cγ2 or Cγ3 region alone, but required the presence of both regions for binding. The RF binding to solid-phase IgG were poorly inhibited by the IgG3 subclass and strongly inhibited by staphylococcal protein A (SPA) (42 kD), and fragment D of SPA (7 kD), indicating that the binding site is most likely the same as the Ga antigenic determinant described for IgM-RF, and is in the same location as the site on IgG that binds SPA. pH titration studies of the RF binding to IgG indicated the involvement of histidine and lysine or tyrosine side chains. Chemical modification studies showed the histidines were involved on the Fc side of the interactions, and tyrosines were involved on both the antigenic and antibody sides of the interactions. Lysines were not involved. The above information, and the knowledge of the number and position in space of the amino acid residues involved in the Cγ2-Cγ3 interface region of IgG, the binding site for SPA, and the amino acid substitutions in IgG3 that account for its inability to bind protein A, allowed the identification of the site on IgG that bind IgG-RF. This binding site involves some of the same amino acid side chains, His 435, Tyr 436, and one or both His 433 and 310, and is in the same location as the site that binds SPA. The same site is likely to be a common antigenic determinant for other RF. Furthermore, the described molecular mimicry suggests a biological relationship between bacterial Fc-binding proteins and the production of RF in rheumatoid arthritis.

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