

# STUDIES ON THE ANTIGENIC DETERMINANTS IN THE SELF-ASSOCIATION OF IgG RHEUMATOID FACTOR\*

BY FRANCIS A. NARDELLA, DAVID C. TELLER, AND MART MANNIK

*From the Division of Rheumatology, Department of Medicine, RG-20, and Department of Biochemistry, SJ-70, University of Washington, Seattle, Washington 98195*

Intermediate complexes, sedimenting between the 6.6S and 19S components of normal serum on sedimentation velocity ultracentrifugation, were first described by Kunkel et al. (1) in the serum of some patients with rheumatoid arthritis. These complexes consisted of IgG (1, 2) and had rheumatoid factor activity (2). In addition, intermediate- and faster-sedimenting complexes were identified in the synovial fluid of some patients with rheumatoid arthritis (3, 4). Previous work from this laboratory using sedimentation equilibrium ultracentrifugation (5, 6) demonstrated that the intermediate complexes isolated from the plasma of three patients with rheumatoid arthritis were composed of self-associated IgG rheumatoid factor (IgG-RF).<sup>1</sup> Dimers of self-associated IgG-RF were observed that underwent concentration-dependent polymerization. A model was proposed for self-association that accounted for the stability of dimers by the formation of two antigen-antibody bonds between two IgG-RF molecules. The association constant for dimer formation was not experimentally determined, but was calculated ( $10^{10}$  liter/mol) as the square of the measured association constant of each individual bond formed between the F(ab')<sub>2</sub> fragment of IgG-RF and normal IgG, which was  $\sim 10^5$  liter/mol. Undoubtedly, the calculated value would be too high because of energy required for ring closure. The polymerization of dimers to higher molecular forms had an association constant also on the order of  $10^5$  liter/mol.

This report describes studies on the number, location, and other characteristics of the antigenic determinants for the self-association of IgG-RF using the IgG-RF isolated from one patient with rheumatoid arthritis as a model system.

## Materials and Methods

*Affinity Isolation of IgG-RF.* The plasma from patient J.J. was obtained and stored as described previously (5). IgG-RF was isolated by affinity chromatography on either human Cohn fraction II (Miles Laboratories, Inc., Elkhart, Ind.) or monomeric IgG agarose columns. Monomeric IgG was obtained by gel filtration of human Cohn fraction II on Sephadex G-200 columns (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) equilibrated

\* Supported by research grants AM-12849 from the National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health; the Townsend-Henderson Fund; and the Washington State Eagles/Arthritis Fund. Presented in part at the American Rheumatism Association meeting in June 1979 and at the International Congress on Rheumatoid Arthritis, Tokyo, 1980.

<sup>1</sup> *Abbreviations used in this paper:* IgG-RF, IgG-rheumatoid factor; IgM-RF, IgM rheumatoid factor; R & A IgG, reduced and alkylated IgG; M<sub>N</sub>, number average molecular weight; M<sub>w</sub>, weight average molecular weight; M<sub>z</sub>, Z average molecular weight; M<sub>1</sub>, molecular weight of the smallest major component; PBS, phosphate-buffered saline (0.05 M phosphate, 0.15 M NaCl, pH 7.3); SDS, sodium dodecyl sulfate.

in borate buffer (0.2 M borate, 0.15 M NaCl, pH 8.0) at 4°C. 1–3 ml of J.J. plasma was applied to affinity columns containing 50-ml packed vol of immunoabsorbent previously prepared by covalently coupling 700–1000 mg of IgG to Sepharose CL-4B (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc.) after cyanogen bromide activation (7). These columns were equilibrated at 4°C in high-ionic-strength borate buffer (0.02 M borate, 0.5 M NaCl, pH 8.8) to minimize the nonspecific adsorption of nonrheumatoid factor IgG (8). The rheumatoid factor was eluted with acetate buffer (0.1 M acetate, 0.15 M NaCl, pH 3.5) after washing the columns with ~400 ml of starting buffer. Before use, columns were cycled through the starting and eluting buffers until the peak absorbance at  $A_{280 \text{ nm}}$  of the acid cycle was <0.003. The pH 3.5 acetate eluates were pooled and concentrated to ~1 mg/ml using stirred, positive-pressure concentrators with Diaflow PM-10 membranes (Amicon Corp., Scientific Sys. Div., Lexington, Mass.) and dialyzed into the appropriate buffers. These preparations contained <5% IgM and IgA, determined by radial immunodiffusion (Immunoplate III; Hyland Division of Travenol Laboratories, Inc., Costa Mesa, Calif.); and further purification was not performed before enzymatic digestion.

The complexes isolated by affinity chromatography showed similar stoichiometries of interaction and association constants for dimer polymerization (data not shown) as found in a previous study of the intermediate complexes of this patient where the isolation was carried out by gel filtration chromatography.

*Preparation of Proteolytic Fragments of Normal IgG and IgG-RF.* J.J. Fab and J.J. Fc fragments were prepared from the affinity-isolated complexes by papain digestion (9). Separation of the Fab and Fc was achieved by passage of the digestion mixture, previously dialyzed against the starting Tris buffer (0.1 M Tris-HCl, pH 8.6), over a cellulose DE-52 (Whatman Chemicals, Div. W & R Balston Ltd., Maidstone, Kent, England) ion-exchange column. The Fab fragments emerged with the starting buffer, and the Fc fragments were eluted from the column with a linear sodium chloride gradient (0–0.5 M) in the starting buffer. The monomeric J.J. Fab and J.J. Fc fragments were obtained by separate gel filtration runs on a Sephadex G-100 column (Pharmacia Fine Chemicals, Div. of Pharmacia) equilibrated with phosphate-buffered saline (PBS; 0.05 M phosphate, 0.15 M NaCl, pH 7.3).

Normal Fc and Fc' fragments were prepared from human Cohn fraction II. This material was first purified by ion-exchange chromatography using a DE-52 column equilibrated in Tris buffer. The fallthrough peak was pooled, concentrated, and papain-digested. Separation of Fab and Fc fragments was achieved as described above. Normal Fc' fragments emerged after the Fc fragments with the sodium chloride gradient. The Fc' peak was pooled, concentrated, and gel-filtered on a Sephadex G-100 column equilibrated with PBS to obtain monomeric material.

Sephadex G-100 gel filtration of the Fc-containing peak, obtained with a sodium chloride gradient from the cellulose DE-52 column, yielded intact Fc as well as an intermediate fragment termed Fc<sub>i</sub>, which eluted after the Fc fragments and before the Fc' fragments. This fragment formed a precipitin line with isolated, specific rabbit antibodies to human C $\gamma$ 3 domain, but did not form a precipitin line with isolated, specific rabbit antibodies to human C $\gamma$ 2 domain (both antisera were kindly supplied by Dr. William Arend, Seattle Veterans Administration Hospital, Seattle, Wash.). On sodium dodecyl sulfate (SDS)-polyacrylamide slab gel electrophoresis (10) the fragment consisted of two polypeptide chains of unequal molecular weight, held together by noncovalent forces. The large chain had a relative 26,000 mol wt and the smaller one, 11,000 mol wt. On high-speed sedimentation equilibrium ultracentrifugation at neutral pH, the preparation was homogeneous with a 38,000 mol wt. This information collectively indicated that Fc<sub>i</sub> is composed of two C $\gamma$ 3 domains and one intact C $\gamma$ 2 domain.

pFc' fragments were prepared by pepsin digestion of human Cohn fraction II (11) and subsequent Sephadex G-100 gel filtration of the digestion mixture. J.J. F(ab')<sub>2</sub> was prepared from the affinity-isolated complexes by pepsin digestion (11) and subsequent Sephadex G-200 gel filtration with the column equilibrated in acetate buffer. Rabbit Fc fragments were prepared and isolated from rabbit Cohn fraction II (Miles Laboratories, Inc.) by the method of Porter (9). The immunologic purity of all the fragments was verified in precipitin analysis by double-diffusion in 2% agarose using appropriate antisera.

*Other Protein Preparations.* Human, rabbit, and goat monomeric IgG were obtained by gel

filtration of human, rabbit, and goat Cohn fraction II (Schwarz/Mann, Div. Becton, Dickenson & Co., Orangeburg, N. Y.) on a Sephadex G-200 column equilibrated with PBS.

An IgG3 myeloma protein was isolated from the plasma of patient D.E. by preparative starch-block electrophoresis (12). The peak fractions of the myeloma protein were pooled, concentrated, and gel-filtered on a Sephadex G-200 column equilibrated with PBS. The preparation consisted only of IgG3 as determined by Ouchterlony analysis using precipitating IgG-subclass-specific antisera (Central Laboratory of the Netherlands, Red Cross Blood Transfusion Service, Amsterdam, The Netherlands).

Purified J.J. Fab was treated with neuraminidase (Worthington Biochemical, Corp., Freehold, N. J.) using the method of Hymes et al. (13). Sialic acid determinations before and after neuraminidase treatment were done by the method of Aminoff (14).

Reduced and alkylated (R & A IgG) was produced by the method of Fleischman et al. (15). SDS-polyacrylamide slab gel electrophoresis using 10% polyacrylamide gels verified the splitting of the interchain disulfide bonds.

*Analytical Ultracentrifugation.* High-speed sedimentation ultracentrifugation experiments were performed with a Beckman Model E analytical ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.), using the Rayleigh Optical System. The camera lens for the Rayleigh optics was focused at the two-thirds plane of the cell (16). All experiments were done at 20°C in PBS using Yphantis 6-channel cells. The partial specific volume of all studied components was assumed to be 0.738 ml/g (17). The interference fringe patterns at sedimentation equilibrium and at the end of each run for baseline determinations were recorded on photographic plates (Spectroscopic II; Eastman Kodak Co., Rochester, N. Y.). Programs and apparatus similar to that described by DeRosier et al. (18) were employed to read the plates. Editing of the raw data obtained from the plate-reading program as well as the calculation of the point by point number average molecular weight ( $M_N$ ), weight average molecular weight ( $M_W$ ), and Z average molecular weight ( $M_Z$ ) were accomplished by programs previously described (19, 20) using PDP-12 computer (Digital Equipment Corporation, Marlboro, Mass.). Association constants for the interaction of J.J. F(ab')<sub>2</sub> or J.J. Fab with other components were obtained by the programs of Behnke et al. (21). The presence or absence of molecular complexes formed as a result of the interaction of J.J. F(ab')<sub>2</sub> or J.J. Fab with other components was determined by plotting the molecular weight averages against the reciprocal of the next lower molecular weight averages in allowed-space graphs (20). If, in this analysis, the plotted molecular weight averages fell in the allowed space for a proposed molecular species, and the stoichiometry of interaction was known or could be predicted, the presence or absence of a proposed species could be determined. Further proof of the existence of a molecular species was obtained by determining the goodness of fit of the sedimentation equilibrium data to a proposed interaction stoichiometry (21). The presence of a molecular species had to be verified by both methods before its existence was accepted.

## Results

*Molecular Weight Averages and Molecular Weight Homogeneity of the Preparations Studied in Interacting Mixtures.* Each protein preparation was characterized by high-speed sedimentation equilibrium ultracentrifugation before examination of their interactions. Table I is a listing of the  $M_N$ ,  $M_W$ ,  $M_Z$ , and  $M_1$ , referring to the smallest major component determined by the methods of Teller et al. (19).  $M_1$  values were used in the analysis of the sedimentation equilibrium data for complex formation for obtaining association constants by the method of Behnke et al. (21).  $M_N$  is a simple arithmetic average;  $M_W$  and  $M_Z$  are progressively weighted for the molecular weights of the various molecular species present. For a homogeneous macromolecular solution,  $M_N = M_W = M_Z$ , and for a heterogeneous macromolecular solution,  $M_N < M_W < M_Z$ . The small differences in the molecular weight averages of the used materials demonstrate the monomeric nature of the individual preparations.

*Antigenic Valence in Self-Associated IgG-RF.* The antigenic valence for IgG-RF in self-

TABLE I  
Molecular Weights of the Preparations Studied in Interacting Mixtures, Determined by High-Speed Sedimentation Equilibrium Ultracentrifugation

Preparation	Molecular weights			
	$M_N$	$M_w$	$M_z$	$M_1^*$
	$\times 10^{-3} \text{ g/mol} \pm SD$			
Normal human IgG	151.6 $\pm$ 2.7	155.5 $\pm$ 3.4	161.5 $\pm$ 6.0	144.6 $\pm$ 0.2
Human IgG3 $\lambda$	154.7 $\pm$ 0.7	156.4 $\pm$ 0.5	157.9 $\pm$ 3.0	151.2 $\pm$ 2.8
R & A human IgG	150.9 $\pm$ 1.5	152.1 $\pm$ 0.5	153.9 $\pm$ 0.6	149.2 $\pm$ 1.1
Rabbit IgG	147.0 $\pm$ 5.4	150.0 $\pm$ 3.5	153.7 $\pm$ 2.0	145.0 $\pm$ 1.5
Goat IgG	151.5 $\pm$ 1.8	152.4 $\pm$ 0.5	154.4 $\pm$ 4.4	150.9 $\pm$ 0.8
J.J. F(ab) <sub>2</sub>	101.2 $\pm$ 5.1	104.2 $\pm$ 3.6	107.5 $\pm$ 3.7	97.0 $\pm$ 2.9
J.J. Fab	52.3 $\pm$ 1.4	52.8 $\pm$ 0.9	53.4 $\pm$ 1.1	51.4 $\pm$ 1.1
Neuraminidase-treated J.J. Fab	50.1 $\pm$ 1.6	50.7 $\pm$ 0.8	51.2 $\pm$ 0.9	49.0 $\pm$ 1.4
Normal human Fab	47.6 $\pm$ 0.4	47.9 $\pm$ 0.2	48.3 $\pm$ 0.5	46.7 $\pm$ 0.3
J.J. Fc	56.5 $\pm$ 2.0	59.6 $\pm$ 1.6	64.4 $\pm$ 1.7	53.0 $\pm$ 2.2
Normal human Fc	52.7 $\pm$ 0.2	53.9 $\pm$ 1.6	56.1 $\pm$ 0.4	50.9 $\pm$ 0.7
Normal human Fc <sub>i</sub>	38.2 $\pm$ 0.2	38.4 $\pm$ 0.6	39.1 $\pm$ 0.2	38.1 $\pm$ 0.2
Normal human Fc'	22.0 $\pm$ 0.3	22.3 $\pm$ 0.7	23.1 $\pm$ 1.9	21.7 $\pm$ 0.2
Normal human pFc'	24.7 $\pm$ 0.1	25.6 $\pm$ 0.2	27.1 $\pm$ 0.8	23.2 $\pm$ 0.6
Rabbit Fc	52.9 $\pm$ 1.5	53.0 $\pm$ 0.2	54.2 $\pm$ 1.6	53.3 $\pm$ 2.3

\*  $M_1$  refers to the smallest major component determined by the methods described by Teller et al. (19).

association must be more than one, or self-association would terminate at the dimer stage. In addition, the structural symmetry of IgG predicts an antigenic valence of two. To examine this question experimentally, the antigenic valence of human and rabbit IgG, human and rabbit Fc, human Fc<sub>i</sub> and J.J. Fc were compared by studying their interaction with J.J. Fab in molar excess to assure interaction with all available antigenic determinants. Fig. 1 illustrates the allowed-space graphs for these interactions. As illustrated, in the cases of the interaction of J.J. Fab with intact normal human IgG, rabbit IgG, and Fc<sub>i</sub> (Fig. 1 A, B and D, respectively), the data points lie almost entirely in the space for one-to-one complex formation with very few data points in the allowed space of two-to-one complex formation. However, when J.J. Fab was interacted with normal Fc or J.J. Fc (Fig. 1 E and F), the data points occupied the allowed spaces for both one-to-one and two-to-one complex formations. As a specific example, Fig. 1 A shows the allowed space graph for interaction of J.J. Fab and normal IgG. If no complexes were formed, all points would lie along the straight line between the  $M_1$  molecular weight values for J.J. Fab and IgG. If only 1:1 complexes were formed, then points should also be in the triangle formed by the straight lines joining the  $M_1$  molecular weights of J.J. Fab, IgG, and [Fab]<sub>1</sub> IgG. Similarly, if both 2:1 and 1:1 complexes were formed points should lie in the space defined by lines joining the  $M_1$  molecular weights of Fab, [Fab]<sub>1</sub>, IgG and [Fab]<sub>2</sub> IgG as well as in the space of 1:1 complex formation just described. The data points lie almost entirely in the space defined by the straight and curvilinear lines joining the  $M_1$  molecular weights of J.J. Fab, IgG, and a 1:1 complex of J.J. Fab and IgG. The

## SELF-ASSOCIATION OF IgG-RHEUMATOID FACTOR

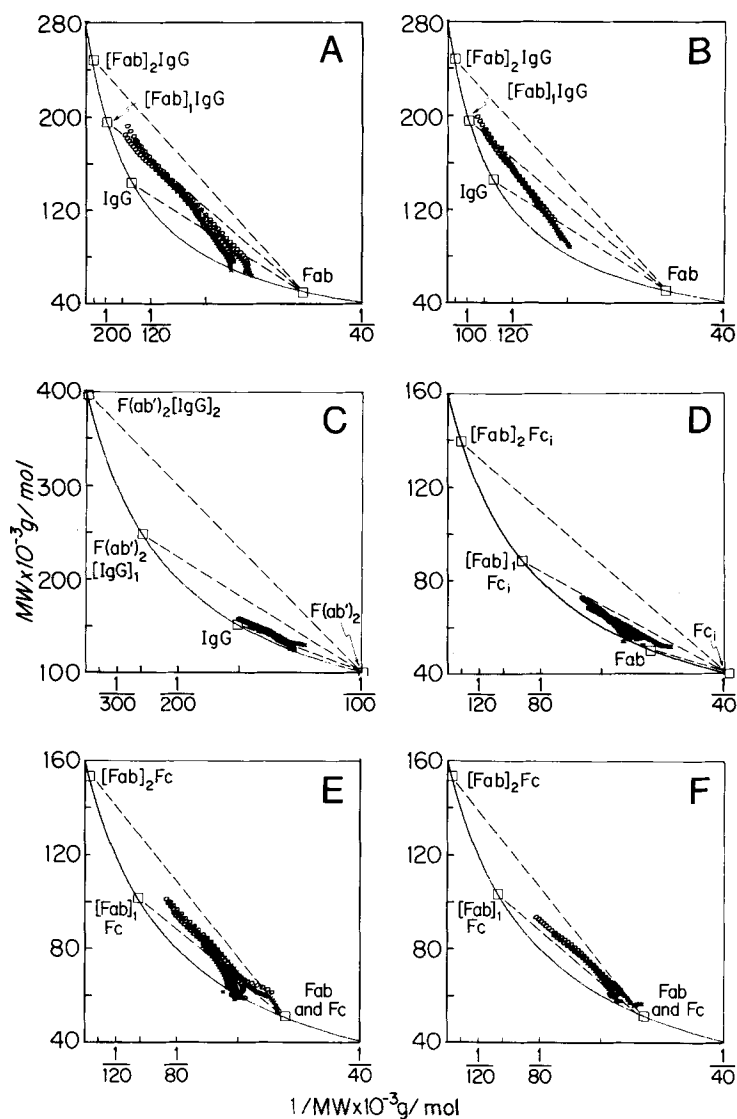


FIG. 1. Allowed-space graphs of the molecular weight ( $MW$ ) averages determined by high-speed sedimentation equilibrium ultracentrifugation for mixtures of J.J. Fab and J.J.  $F(ab)_2$  with various IgG molecules or fragments as antigens. (A) J.J. Fab and normal human IgG, (B) J.J. Fab and rabbit IgG, (C) J.J.  $F(ab)_2$  and goat IgG, (D) J.J. Fab and  $Fc_1$ , (E) J.J. Fab and normal human  $Fc$ , (F) J.J. Fab and J.J.  $Fc$ . (○)  $M_z$  vs.  $1/M_w$ ; (×)  $M_w$  vs.  $1/M_n$  plots as printed by the computer. In this analysis, the molecular weight averages were plotted on the y-axis and the next lower average on the x-axis. The curved line represents the locus of homogeneous materials. The molecular weight averages of the interactants, run separately, would lie about their respective molecular weights indicated on the curved lines by the squares. In the mixtures, if no complexes were formed, all data points would lie along the straight line joining their respective molecular weights. If only 1:1 complexes were formed, then some points would lie in the triangle formed between the molecular weights of the reactants and the 1:1 complex. For 1:1 and 2:1 complex formation, the data points should, in addition, be seen in the next higher triangular space formed by the molecular weights of the 2:1 complex, 1:1 complex, and lowest molecular weight reactant.

conclusions derived from allowed-space graph analyses were corroborated by the good fits of the stoichiometries suggested by the allowed-space graphs to the sedimentation equilibrium data using the methods of Behnke et al. (21). No interaction between J.J. F(ab')<sub>2</sub> and goat IgG was observed with these methods (Fig. 1C). The results indicate that Fc fragments have an antigenic valence of two, but intact IgG molecules have only one antigenic determinant functionally available for interaction with J.J. Fab (Table II).

*Absence of a Unique Antigenic Determinant in Self-Associated J.J. IgG-RF.* The preferential self-association of IgG-RF to form dimers can be explained entirely by the thermodynamics of two antigen-antibody bonds as originally postulated (5, 6). An alternative explanation of this, however, could be the presence of unique antigenic determinants on the IgG-RF that would allow the formation of high-energy bonds

TABLE II  
Summary of the Results of the Allowed-Space Graph Analysis of High-Speed Sedimentation Equilibrium Ultracentrifugation Data for the Interactions between J.J. Fab or F(ab')<sub>2</sub> and Various IgG Molecules or Fragments as Antigens

Interaction*	Presence of indicated molecular species formed between J.J. Fab or F(ab') <sub>2</sub> and antigen		Concluded antigenic valence
	1:1	2:1	
J.J. Fab + normal human IgG	Yes	No	1
J.J. Fab + R & A IgG	Yes	No	1
J.J. Fab + rabbit IgG	Yes	No	1
J.J. F(ab') <sub>2</sub> + Goat IgG	No	No	0
J.J. Fab + Fc <sub>1</sub>	Yes	No	1
J.J. Fab + normal human Fc	Yes	Yes	2
J.J. Fab + J.J. Fc	Yes	Yes	2
J.J. Fab + rabbit Fc	Yes	Yes	2

\* The J.J. Fab fragments were in 1.5 or greater molar excess of each antigen. The goat IgG was in 2-fold molar excess of J.J. F(ab')<sub>2</sub>.

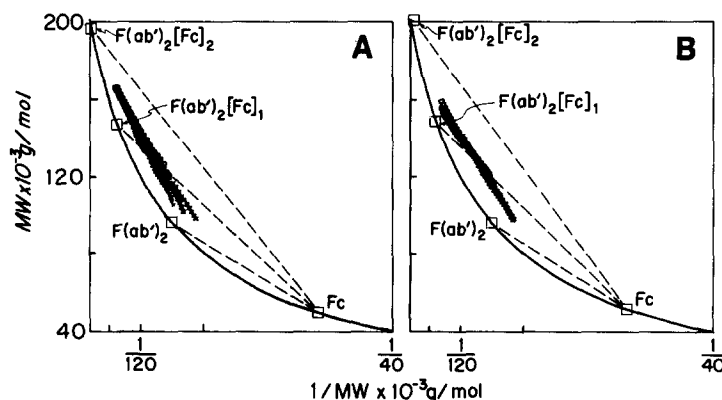


FIG. 2. Allowed-space graphs of molecular weight (MW) averages for the interactions of (A) J.J. F(ab')<sub>2</sub> and normal human Fc, and (B) J.J. F(ab')<sub>2</sub> and J.J. Fc. See legend to Fig. 1 for details on the nature of the plots.

TABLE III  
*Equilibrium Constants and Free Energies for the Interactions between J.J. F(ab')<sub>2</sub> and Various Antigens*

Interaction	K <sub>1</sub> *	-ΔG°	K <sub>2</sub> *‡	-ΔG°
	liter/mol	kcal/mol ± SD	liter/mol	kcal/mol ± SD
J.J. F(ab') <sub>2</sub> + normal human Fc	1.09 × 10 <sup>5</sup>	6.76 ± 0.70	2.29 ± 10 <sup>5</sup>	7.19 ± 1.02
J.J. F(ab') <sub>2</sub> + J.J. Fc	2.42 × 10 <sup>5</sup>	7.22 ± 0.10	0.66 × 10 <sup>5</sup>	6.46 ± 0.15
J.J. F(ab') <sub>2</sub> + rabbit Fc	1.12 × 10 <sup>5</sup>	6.77 ± 0.61	3.41 × 10 <sup>5</sup>	7.42 ± 1.15
J.J. F(ab') <sub>2</sub> + normal human IgG	3.84 × 10 <sup>5</sup>	7.49 ± 0.59	0.81 × 10 <sup>5</sup>	6.58 ± 0.43
J.J. F(ab') <sub>2</sub> + human myeloma IgG3	2.05 × 10 <sup>5</sup>	7.13 ± 0.81	0.10 × 10 <sup>5</sup>	5.44 ± 0.36

\* The values of equilibrium association constants (K) were calculated from mean values of free energies (-ΔG°).

‡ K<sub>2</sub> is taken to represent the interaction of the unfilled F(ab')<sub>2</sub> combining site with antigen because these experiments were carried out in 1.5 or greater molar excess of antigen. In addition, for two identical binding sites, the free energy of the first binding should be ≈0.8 kcal greater than the second as occurs in rows 1, 2, and 4, i.e., ΔG<sub>1</sub> = ΔG<sub>2</sub> - RT(ln 4) because K<sub>1</sub> = 4K<sub>2</sub>.

during self-interaction and lower energy bonds with interaction with normal IgG. To explore this possibility, the strength of interaction of J.J. F(ab')<sub>2</sub> with normal human Fc and J.J. Fc were compared using 1.5:1 or greater molar excess of Fc. The binding energies for J.J. Fab and various Fc preparations could not be obtained because the similarities of their molecular weights did not allow their respective concentrations to be determined by the employed methods. The allowed space graphs (Fig. 2 A and B) show that the products of interaction, i.e., both 1:1 and 1:2 complexes, between J.J. F(ab')<sub>2</sub> and normal human Fc were similar to those formed in interaction with J.J. Fc. In addition, the free energies of these interactions were comparable (Table III), indicating that the antigenic determinants present on J.J. IgG-RF are not unique.

*Location of the Antigenic Determinants in Self-Association of IgG-RF.* The location of the antigenic determinants for self-associated IgG-RF may have considerable influence on the biologic properties of these immune complexes and this information may assist in the definition of the actual antigenic determinant. For this purpose the interaction of J.J. Fab with pepsin-derived Fc fragments (pFc') and Fc' fragments comprising the Cγ3 region were studied. allowed-space graphs (Fig. 3 A and B) show that only small amounts of 1:1 complex formation occurred with both pFc' and Fc'. The free energies for these interactions were quite low, ~5 kcal/mol (Table IV). The possibility existed that this weak binding represented nonspecific protein-protein interaction or a specific interaction of J.J. Fab with part of the rheumatoid factor antigenic determinant or with the antigenic determinant that loses part of the necessary conformation with loss of the Cγ2 region. To explore this further, the interaction was studied between normal Fab and pFc', using exactly the same initial loading concentrations and molar ratios (1.2:1, Fab:pFc') as used for the interaction of J.J. Fab and pFc'. The results of this experiment (allowed-space graph not shown) indicated that small amounts of 1:1 complex were formed and the energy of interaction was the same (4.91 ± 0.25 kcal/mol; see Table IV) as observed for J.J. Fab and pFc'. These results indicate that the minimal interaction between J.J. Fab and pFc' or Fc' are a result of nonspecific protein-protein interactions and do not represent rheumatoid factor binding to its specific antigenic determinant. Therefore, the antigenic determinant for IgG-RF is not present on the Cγ3 domain, but is either on the Cγ2 domain or requires the presence of both Cγ2 and Cγ3 domains for expression. That J.J. Fab formed only 1:1 complexes with the Fc<sub>i</sub> fragment (Tables II and IV) further corroborates the above

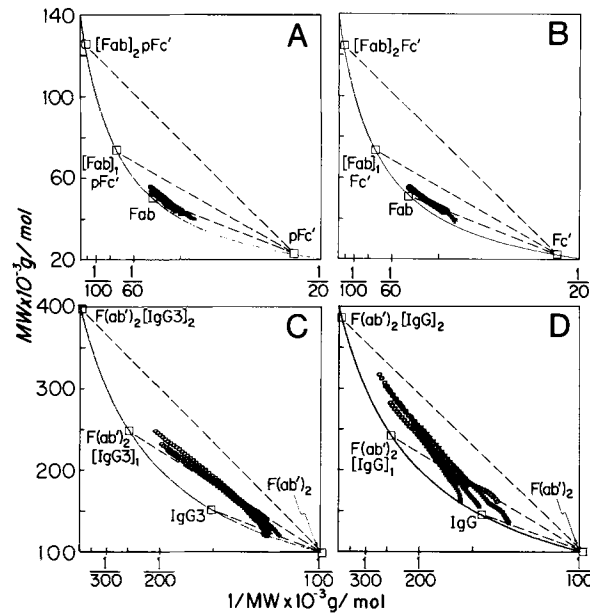


FIG. 3. Allowed-space graphs of the molecular weight (MW) averages for the interactions of (A) J.J. Fab and pFc', and (B) J.J. Fab and Fc'; (C) J.J. F(ab')<sub>2</sub> and human myeloma IgG3, and (D) J.J. F(ab')<sub>2</sub> and normal human IgG. See legend to Fig. 1 for details on the nature of the plots.

TABLE IV  
Equilibrium Constants and Free Energies for the Interactions between J.J. Fab or Normal Fab and Various Antigens

Interaction	K*	-ΔG°
	liter/mol	kcal/mol ± SD
Normal Fab + normal human pFc'	$4.53 \times 10^3$	$4.91 \pm 0.25$
J.J. Fab + normal human pFc'	$5.55 \times 10^3$	$5.02 \pm 0.33$
J.J. Fab + normal human Fc'	$6.48 \times 10^3$	$5.11 \pm 0.32$
J.J. Fab + normal human Fc <sub>i</sub>	$0.40 \times 10^6$	$6.17 \pm 0.04$
J.J. Fab + normal human IgG	$1.80 \times 10^6$	$7.05 \pm 0.88$
J.J. Fab <sub>N</sub> § + normal human IgG	$0.99 \times 10^6$	$6.70 \pm 0.13$
J.J. Fab + R & A human IgG	$1.47 \times 10^6$	$6.93 \pm 0.22$

\* The values of equilibrium association constants (K) are averages obtained from mean values of free energies (-ΔG°).

§ Neuraminidase-treated J.J. Fab.

interpretations because the Fc<sub>i</sub> is composed of an intact heavy chain structure on one side and is missing the Cγ2 domain on the other.

The so-called Ga antigen (22) is an antigenic determinant for some IgM-RF, present on the IgG1, -2, and -4 subclasses but not on IgG3. This determinant was not present on the Cγ3 domain and therefore was thought to exist on the Cγ2 domain (23). For this reason, the interaction of J.J. F(ab')<sub>2</sub> with an IgG3 myeloma protein was carried out. The interaction occurred with the formation of similar molecular weight products (Fig. 3C and D allowed-space graphs) and strengths of binding (Table III) as the interactions of J.J. F(ab')<sub>2</sub> and normal human IgG, indicating that the antigenic determinants for J.J. IgG-RF is not the Ga antigen.



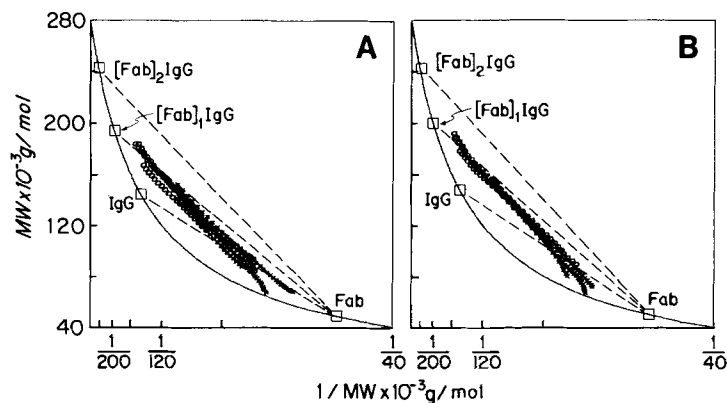


FIG. 4. Allowed-space graphs of the molecular weight (MW) averages for the interactions of (A) neuraminidase-treated J.J. Fab and normal human IgG, and (B) J.J. Fab and reduced and alkylated IgG. See Fig. 1 legend for details on the nature of the plots.

*Effect of Neuraminidase Treatment on IgG-RF Antibody Activity.* Neuraminidase treatment of the  $F(ab')_2$  fragments, prepared from the intermediate complexes from the serum of a patient with rheumatoid arthritis and Hodgkin's disease, abolished their ability to form complexes with normal IgG as studied by sedimentation velocity analytical ultracentrifugation (13). To examine this possibility in J.J. IgG-RF, neuraminidase digestion of J.J. Fab was carried out. Before neuraminidase digestion, the sialic acid content was 1.24 on J.J. IgG-RF, 0.43 on J.J. Fab, and after digestion, 0, as expressed in moles per mole. No change in rheumatoid factor activity was seen, as shown by formation of the expected 1:1 complexes between neuraminidase-treated J.J. Fab and normal IgG (Fig. 4A) as well as by the calculated association constant and free energy (Table IV). In addition, neuraminidase digestion of isolated J.J. IgG-RF did not alter its self-association characteristics from those reported (5, 6). The stoichiometries and association constants for dimer polymerization were the same as for unaltered J.J. IgG-RF (data not shown).

*Effect of Reduction and Alkylation of the Interchain Disulfide Bonds on the Antigenic Activity of Normal IgG in Its Interaction with J.J. Fab.* If the  $C\gamma 2$  region is involved in the antigenic determinant for self-association of IgG-RF, then reduction and alkylation of the interchain disulfide bonds might alter this binding because the flexibility of the hinge region is increased by this manipulation (24, 25). This increased hinge-region flexibility, for example, might be expected to make both antigenic determinants present on normal IgG accessible, only one of which is functionally available in the intact molecule. To explore this possibility, the interaction of J.J. Fab and R & A IgG was studied. The allowed-space graph for this interaction (Fig. 4B) shows molecular weight products similar to the interaction of J.J. Fab and unaltered IgG, i.e., the formation of only 1:1 complexes and thus an antigenic valence of one (Table II). In addition, the association constant and free energy were comparable with the values obtained with unaltered IgG (Table IV), indicating that no measurable perturbation of the rheumatoid factor bond occurred with the R & A IgG serving as the antigen. Furthermore, a sample of the isolated self-associated J.J. IgG-RF was reduced, alkylated, and examined by sedimentation equilibrium ultracentrifugation. The analysis of the results yielded stoichiometries and association constants for polymeri-

zation of dimers (data not shown) similar to those previously reported (5, 6). These findings collectively indicate that relaxation of the hinge region of IgG-RF by the cleavage of the interchain disulfide bonds does not alter the self-association of IgG-RF. These findings are in agreement with Kunkel et al. (1) who found no alteration in the sedimentation velocities of intermediate complexes that had been reduced and alkylated.

### Discussion

The antigenic valence of human IgG and its fragments for interaction with IgG-RF was determined by sedimentation equilibrium ultracentrifugation, utilizing a molar excess of the Fab fragments of IgG-RF. The antigenic valence was two for normal human Fc fragments, Fc fragments of self-associating IgG-RF, and rabbit Fc fragments. This finding is consistent with the fact that the Fc fragments are composed of the carboxy-terminal halves of both gamma chains in the IgG molecule. Of considerable interest was the finding of a functional valence of one for the intact normal IgG molecule. Because on a structural basis the valence should be two, and, in fact, was two in the Fc fragments, this finding implies that when one antigenic determinant is occupied by the Fab fragment of the IgG-RF, the other antigenic determinant becomes functionally unavailable. The mechanism for this has not been determined. It is possible, however, that the binding of the first Fab fragment of IgG-RF to one antigenic determinant rotates the Fab regions of IgG about the hinge region so that the other antigenic determinant is no longer accessible. Of note is that the antigenic valence of IgG for interaction with Fab $\mu$  derived from IgM rheumatoid factor was also one (26).

The antigenic valence of IgG-RF in self-association must be two, even though it was not possible to verify this experimentally. If the antigenic valence of IgG-RF were one, the self-association would terminate at dimer formation. Previously published studies clearly showed that the self-associated dimers underwent further concentration-dependent polymerization (5, 6), thereby indicating an antigenic valence of two. These observations suggest that in the formation of IgG-RF dimers, held together by two antigen-antibody bonds, the Fab fragments of both molecules are sufficiently rotated to expose both existing antigenic determinants. This could result from the expected high (but not measured) free energy of the two antigen-antibody bonds formed in dimerization of IgG-RF. These suggestions could be verified by high resolution electron microscopy using techniques similar to Seegan et al. (25).

The formation of stable dimers in the self-association of IgG-RF could entirely be explained by thermodynamics of the formation of two antigen-antibody bonds, as previously concluded (5, 6). A preferential self-association, however, could result in part or in whole from the presence of unique antigenic determinants on the Fc regions of IgG-RF, allowing for high energy bonds with self-interaction and low energy bonds with interaction with non-rheumatoid factor IgG. Therefore, the interaction of F(ab')<sub>2</sub> fragments of IgG-RF with its own Fc fragments and with normal Fc fragments were compared. The similarity of the formed complexes and the similarity of the association constants definitively excluded the presence of unique antigenic determinants on the IgG-RF molecules and reaffirmed the two antigen-antibody bond hypothesis as the basis for stable dimer formation of these antibodies.

The precise identification and localization of the antigenic determinant or deter-

minants for self-association of IgG-RF would be of interest in understanding the nature of the reaction as well as the biologic properties of these complexes, including complement fixation and interaction with Fc receptors of various cell populations. The Fab fragments of IgG-RF interacted very weakly with Fc' and pFc' fragments (C $\gamma$ 3 regions). This interaction, however, was similar to the interaction of normal Fab and pFc', suggesting that the small degree of interaction observed with J.J. Fab was most likely a nonspecific protein-protein interaction. Thus, these results indicate that the antigenic determinant is not located on the C $\gamma$ 3 domain but is located either on the C $\gamma$ 2 domain, or that its expression requires the intactness of both domains. In addition, the binding of the F(ab')<sub>2</sub> fragments of IgG-RF to an IgG3 myeloma protein indicates that the antigenic determinant is not the common Ga antigenic determinant for some IgM-RF, which is present on IgG1, -2 and -4 subclasses, but absent on the IgG3 subclass. Further work is needed to identify the peptide or peptides that constitute the antigenic determinant for IgG-RF, the location of these peptides in the sequence of the  $\gamma$  chains and their location on the three-dimensional models of IgG molecules.

Because the functional valence of normal IgG for interaction with IgG-RF was only one, the possibility existed that reduction and alkylation of the inter-chain disulfide bonds, known to increase the flexibility of the hinge region (24, 25), might make the second antigenic determinant available. This alteration of normal IgG, however, neither altered the antigenic valence nor the free energy of the interaction with Fab fragments of IgG-RF. The relaxation of the hinge region by reduction and alkylation, on the other hand, has been thought to account for the abrogation of complement fixation by molecules altered in this manner when combined with specific antigens (25).

The antigenic valence of one for normal IgG molecules in the interaction with IgG-RF has important implications. As already noted, the further polymerization of self-associated IgG-RF dimers proceeds with free energy comparable to the free energy of binding normal IgG to one antibody combining site of IgG-RF. Therefore, in the presence of abundant normal IgG, the interaction of normal IgG with self-associated IgG-RF dimers by mass action would be preferred. This in turn would effectively terminate further polymerization because the antigenic valence of normal IgG is one, resulting in complexes consisting of two self-associated IgG-RF molecules and two

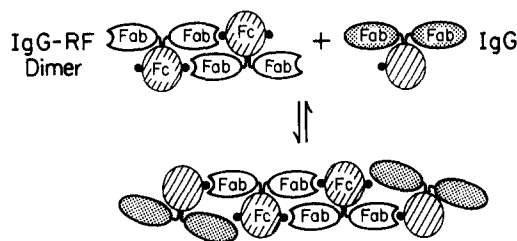


FIG. 5. Schematic representation of the termination of polymerization of IgG-RF dimers by excess normal IgG. Although two antigenic determinants exist on isolated Fc fragments, only one determinant is functionally available on intact IgG for binding to IgG-RF. The binding of IgG-RF to one determinant may rotate the hinge region and thus sterically block the second site, as depicted on this diagram by alteration of the position of Fab arms on IgG after interaction with IgG-RF dimer. On this basis, the presence of excess IgG would terminate further polymerization of self-associated IgG-RF dimers.

normal IgG molecules as depicted in Fig. 5. This complex would have an  $\sim 600,000$  mol wt and a sedimentation coefficient of  $\sim 14S$ , consistent with the so-called intermediate complexes found in patients with rheumatoid arthritis. The termination of high-polymer formation of self-associating IgG-RF by normal IgG also could, in part, explain the finding of smaller immune complexes in serum than in synovial fluids of patients with rheumatoid arthritis by several investigators (3, 4). In turn, the formation of large polymers of self-associating IgG-RF in the synovial tissues or synovial fluid could contribute to the inflammatory events characteristic of rheumatoid synovitis.

### Summary

The number, location, and other characteristics of the antigenic determinants for self-association of IgG-rheumatoid factors (IgG-RF) were examined using the IgG-RF isolated from the plasma of one patient as a model system. Affinity chromatography was employed for isolation of the IgG-RF. Sedimentation equilibrium ultracentrifugation was used to study the various interactions.

The antigenic valence of IgG-RF Fc, normal human Fc, and rabbit Fc fragments was two for the interaction with Fab fragments from IgG-RF, as might be expected from the molecular symmetry of IgG. The antigenic valence of intact normal IgG, however, was only one, indicating that when one of the available antigenic determinants interacted with the Fab fragment of IgG-RF, the other determinant becomes sterically inaccessible. Reduction and alkylation, known to increase the flexibility of the hinge region, did not alter the antigenic valence of IgG for Fab fragments of IgG-RF. The antigenic valence of IgG-RF in self-association could not be experimentally determined but must be two to permit the observed concentration-dependent further polymer formation of IgG-RF dimers.

Unique antigenic determinants on the Fc fragments of IgG-RF were sought and not found, thus reaffirming the formation of two antigen-antibody bonds as the basis for dimerization of IgG-RF molecules.

The pFc' and Fc' fragments, representing C $\gamma$ 3 domains of IgG, failed to show significant interaction with Fab fragments of IgG-RF, indicating that the antigenic determinants were not expressed by the C $\gamma$ 3 regions but are located either on C $\gamma$ 2 region or require intact C $\gamma$ 2 and C $\gamma$ 3 regions for expression. These conclusions were corroborated by the antigenic valence of one for the Fc<sub>i</sub> fragment, a new papain-generated intermediate fragment of Fc, composed of two intact C $\gamma$ 3 domains and one intact C $\gamma$ 2 domain.

Normal IgG, because of its valence of one for interaction with IgG-RF, would effectively terminate further polymerization of IgG-RF dimers. This may well in part explain the finding of smaller IgG-RF complexes in the serum than in synovial fluid of patients with rheumatoid arthritis.

We thank Dr. Fred Myers for providing some of the early preparations used in these studies, Bruce Pieteron, Charles Barber, and Patrick Goldsworthy for technical assistance, and Nisa Rachie for secretarial assistance.

*Received for publication 16 March 1981.*

## References

1. Kunkel, H. G., H. J. Müller-Eberhard, H. H. Fudenberg, and T. B. Tomasi. 1960. Gamma globulin complexes in rheumatoid arthritis and certain other conditions. *J. Clin. Invest.* **40**: 117.
2. Schrohenloher, R. D. 1966. Characterization of the  $\gamma$ -globulin complexes present in certain sera having high titers of anti- $\gamma$ -globulin activity. *J. Clin. Invest.* **45**:501.
3. Hannestad, K. 1967. Presence of aggregated  $\gamma$ -globulin in certain rheumatoid synovial effusions. *Clin. Exp. Immunol.* **2**:511.
4. Winchester, R. J., V. Agnello, and H. G. Kunkel. 1970. Gamma globulin complexes in synovial fluids of patients with rheumatoid arthritis. Partial characterization and relationship to lowered complement levels. *Clin. Exp. Immunol.* **6**:689.
5. Pope, R. M., D. C. Teller, and M. Mannik. 1974. The molecular basis of self-association of antibodies to IgG (rheumatoid factors) in rheumatoid arthritis. *Proc. Natl. Acad. Sci. U. S. A.* **71**:517.
6. Pope, R. M., D. C. Teller, and M. Mannik. 1975. The molecular basis of self-association of IgG-rheumatoid factors. *J. Immunol.* **115**:365.
7. Stage, D. E., and M. Mannik. 1974. Covalent binding of molecules to CNBr-activated agarose: parameters relevant to the activation and coupling reactions. *Biochim. Biophys. Acta.* **343**:382.
8. Nardella, F. A., and M. Mannik. 1978. Nonimmunospecific protein-protein interactions of IgG: Studies of the binding of IgG to IgG immunoabsorbents. *J. Immunol.* **120**:739.
9. Porter, R. R. 1959. The hydrolysis of rabbit  $\gamma$ -globulin and antibodies with crystalline papain. *Biochem. J.* **73**:119.
10. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. **227**:680.
11. Turner, M. W., and H. Bennich. 1968. Subfragments from the Fc fragments of human immunoglobulin G. *Biochem. J.* **107**:171.
12. Kunkel, H. G. 1954. Zone electrophoresis. In *Methods of Biochemical Analysis*. D. Glick, editor. Interscience Publishers, New York. **1**:141.
13. Hymes, A. J., G. L. Mullinax, and T. Mullinax. 1979. Immunoglobulin carbohydrate requirement for formation of an IgG-IgG complex. *J. Biol. Chem.* **254**:3148.
14. Aminoff, D. 1961. Methods for the quantitative estimation of N-acetylneuraminic acid and their application to hydrolysates of sialomucoids. *Biochem. J.* **81**:384.
15. Fleischman, J. B., R. H. Pain, and R. R. Porter. 1962. Reduction of gammaglobulin. *Arch. Biochem. Biophys.* **1** (Suppl.):174.
16. Svensson, H. 1954. The second order aberrations in the interferometric measurement of concentration gradients. *Opt. Acta.* **1**:25.
17. Marler, E., C. A. Nelson, and C. Tanford. 1964. The polypeptide chains of rabbit  $\gamma$ -globulin and its papain-cleaved fragments. *Biochemistry.* **3**:279.
18. DeRosier, D. J., P. Munk, and D. J. Cox. 1972. Automatic method of interference photographs from the ultracentrifuge. *Anal. Biochem.* **50**:139.
19. Teller, D. C., T. A. Horbett, E. G. Richards, and H. K. Schachman. 1969. Ultracentrifuge studies with Rayleigh interference optics. III. Computational methods applied to high-speed sedimentation equilibrium experiments. *Ann. N. Y. Acad. Sci.* **164**:66.
20. Teller, D. C. 1973. Characterization of proteins by sedimentation equilibrium in the analytical ultracentrifuge. *Methods Enzymol.* **27D**:346.
21. Behnke, W. D., D. C. Teller, R. D. Wade, and H. Neurath. 1970. Hybrid formation of carboxypeptidase and fraction II of procarboxypeptidase A. *Biochemistry.* **9**:4189.
22. Allen, J. C., and H. G. Kunkel. 1966. Hidden rheumatoid factors with specificity for native  $\gamma$ -globulins. *Arthritis Rheum.* **9**:758.

23. Natvig, J. B., P. I. Gaarder, and M. W. Turner. 1972. IgG antigens of the C $\gamma$ 2 and C $\gamma$ 3 homology regions interacting with rheumatoid factors. *Clin. Exp. Immunol.* **12**:177.
24. Romares, D. G., C. A. Tilley, M. C. Crookston, R. E. Falk, and K. J. Dorrington. 1977. Conversion of incomplete antibodies to direct agglutinins by mild reduction: evidence for segmental flexibility within the Fc fragment of IgG. *Proc. Natl. Acad. Sci. U. S. A.* **74**:2531.
25. Seegan, G. W., C. A. Smith, and V. N. Schumaker. 1978. Changes in quaternary structure of IgG upon reduction of the interheavy-chain disulfide bond. *Proc. Natl. Acad. Sci. U. S. A.* **76**:907.
26. Stone, M. J., and H. Metzger. 1968. Binding properties of a Waldenström macroglobulin antibody. *J. Biol. Chem.* **243**:5977.