BIOLOGICAL ACTIVITY OF THE CLEAVAGE PRODUCT OF HUMAN IMMUNOGLOBULIN G WITH CYANOGEN BROMIDE*

By MIRA LAHAV,‡ PH.D., RUTH ARNON, PH.D., AND MICHAEL SELA, PH.D.

(From the Section of Chemical Immunology, The Weizmann Institute of Science, Rehovoth, Israel)

(Received for publication 28 December 1966)

The fragments resulting from the enzymic digestion of human immunoglobulin G (IgG) have been extensively studied with respect to immunological properties (for review, see Porter and Press, reference 1). Papain cleaves IgG in the presence of cysteine into three fragments (2–4), two Fab and one Fc (for nomenclature, see reference 5). The Fab fragments, though monovalent, retain full combining specificity for antigen (2), whereas the Fc fragment contains most of the antigenic determinants of IgG. Pepsin cleaves IgG to yield a divalent active fragment $F(ab')_2$, and smaller fragments derived from the Fc portion of the molecule (6). Reduction of IgG under mild conditions yields two types of chains, heavy and light, which can be separated by gel filtration (7). Some of the biological properties of native immunoglobulins, such as complement-binding capacity, skin-fixing ability, and antigenic specificities have been shown to be localized in individual subunits and chains (8–11).

Chemical reagents which cleave selectively peptide bonds adjacent to specific amino acids have been helpful in recent years in structural studies of proteins. Cleavage of methionyl bonds with cyanogen bromide (CNBr) has been the method of preference, as practically no side reactions take place (12). This reagent has been used successfully in the determination of amino acid sequences in protein chains, in the proof of the nonidentity of chains, and in drawing conclusions about protein conformation (literature cited in reference 13). Recently, studies on the amino acid sequence and peptide maps of immunoglobulin chains, using cyanogen bromide cleavage, have been reported (14–18).

Whereas, in studies of the amino acid sequences of proteins, the efficiency of the method depends on the quantitative cleavage of peptide bonds adjacent to all methionine residues in the molecule, for studies of the correlation of biological activity of a protein with its chemical structure, a limited cleavage may be of interest. Previous reports from this laboratory described CNBr

* Supported in part by Agreement 415110 with the National Institutes of Health, United States Public Health Service, Bethesda, Md.

‡ On leave of absence from the Central Laboratories, Sick Fund Organization, Tel Aviv, Israel.

cleavage of rabbit IgG under milder conditions, leading to a preferential destruction of that part of the molecule with which Fc is associated, and to the formation of "5S" fragments which retained antibody activity (13, 19). Such milder conditions were also used in studies of the amino acid composition of different moieties of two antibodies (20).

The present investigation is concerned with similar cleavage of human IgG and indicates that, in resemblance to the rabbit protein, most of the antibody activity of human IgG is retained after cleavage with CNBr. In contrast to the antibody activity, which is known to be located in the Fab fragment, other biological properties which are related to the Fc fragment, such as genetic markers and other antigenic determinants, as well as complement-binding and skin-fixation sites, are essentially lost during the CNBr cleavage.

In accordance with the accepted denotation of $F(ab')_2$ for 5S fragments obtained by pepsin digestion of IgG (5), we denote similar fragments, having two antibody sites, obtained by CNBr cleavage, as $F(ab'')_2$.

Materials and Methods

Immunoglobulin G.—Normal human IgG was prepared from outdated human plasma by precipitation with ammonium sulfate (40% saturation). The washed precipitate was dissolved in water and dialyzed exhaustively against large volumes of 0.15 M NaCl followed by short dialysis against 0.0167 M phosphate buffer, pH 6.3 (during the dialysis fibrinogen precipitated). The clear solution was chromatographed in the same buffer on diethylaminoethylcellulose (Bio-Rad Laboratories, Inc., Richmond, Calif.) according to Levy and Sober (21), and the fraction eluted was concentrated by vacuum dialysis.

Human IgG with antibody activity was prepared in a similar way, starting from serum instead of plasma. Thus, IgG with antistreptolysin activity was isolated from high titer serum of patients suffering from rheumatic fever, and anti-A group IgG was isolated from sera of individuals of O blood group.

The immunoglobulin solutions were stored in the frozen state. The protein concentration was determined spectrophotometrically, assuming a specific extinction coefficient of $E^{1\%}_{1\text{cm}} = 13.8$ at 280 mμ (22).

Cyanogen Bromide Treatment of IgG.—A solution of IgG (about 50 mg/ml) was mixed with an equal volume of a freshy prepared 0.15 M solution of crystalline CNBr (Eastman Kodak Co., Rochester, N. Y.) in 0.1 M HCl. The mixture was allowed to stand in a tightly closed glass-stoppered container at room temperature for 4 hr.

Column Fractionation of the CNBr Digest.—The reaction mixture described above (6–8 ml, containing about 200 mg) was applied to a Sephadex G-100 (Pharmacia, Uppsala, Sweden) column (2.5 × 150 cm) previously equilibrated with 0.05 M HCl. Elution was carried out at 4°C with 0.05 M HCl, and the absorbancy of the effluent solution was measured at 280 mμ.

Amino Acid Analysis.—Amino acid analyses (23) were performed on the Beckman/Spinco automatic amino acid analyzer, model 120B, after hydrolysis under reduced pressure in constant boiling hydrochloric acid (6 N) for 22 hr at 110°C.

Physical Methods.—Spectrophotometric measurements were made on a Zeiss model PMQII spectrophotometer, at approximately 25°, with quartz cells of 1 cm light path.

Sedimentation measurements were carried out in a Spinco model E ultracentrifuge, at 20–22°, with the Schlieren optical system. The samples were sedimented at 59,780 rpm. Measurements were made at various pH values ranging from 2–9, in both aqueous and urea solutions.

Starch gel electrophoresis in 8 M urea formate buffer, was carried out as described by Edelman and Poulik (24).

Free boundary electrophoresis was carried out in a Tiselius apparatus using a 2 ml cell and 0.1 M barbital buffer, pH 8.6, as described by Longsworth (25).

Antisera.—Human IgG (prepared as mentioned above) or human light chains (prepared according to Fleischman et al., reference 26) were used for the preparation of goat antisera. Immunization was accomplished by injection at multiple intradermal sites of a total amount of 30 mg protein in complete Freund's adjuvant (Difco Laboratories, Inc., Detroit, Mich.). Blood was collected from the external jugular vein. Either the antisera or IgG isolated from them as described above for human IgG, were used in the various experiments.

Gel Diffusion.—This was carried out in agar gel in 0.05 M barbital buffer, pH 8.6, according to Ouchterlony (27).

Complement-Binding Activity.—The procedure was essentially according to Mayer et al. (28,29), using lyophilized guinea pig complement and anti-sheep hemolysin (Difco). In a typical experiment various amounts (2–4 mg) of IgG, both native and CNBr-treated, were mixed in the cold with a constant amount (22 $C'H_{50}$ units) of complement. Following incubation for 30 min at 37°C and storage in the cold room overnight, the solutions were diluted to yield a concentration suitable for the estimation of the residual activity of complement.

Antistreptolysin Activity.—This was determined according to Rantz and Randall (30). Solutions of 1 ml containing various amounts (0.1–50 μg) of IgG, both native and subjected to various treatments, were mixed with 0.5 ml of Bacto Streptolysin O Reagent (Difco). Following 15 min at 37°C, 0.5 ml of 5% rabbit erthyrocytes suspension was added and the mixtures were incubated at 37°C for 45 min. The extent of hemolysis was determined from the absorbancy of the supernatant fluids at 541 mμ.

Isohemagglutinin Activity.—This was determined (31), using IgG preparations from a type O individual, and fresh human erythrocytes from type A individuals. IgG preparations, both native and treated, were brought to pH 8.5, and mixed in twofold serial dilutions in saline, with equal volumes of a 2% suspension of erythrocytes. The mixtures were kept at 37°C for 1 hr and centrifuged thereafter for 5 min at 1000 rpm. Hemagglutination was graded macroscopically from 1+ to 3+.

Reverse Passive Cutaneous Anaphylaxis (RPCA).—RPCA experiments were performed in guinea pigs according to Ovary (32), using human IgG and CNBr-treated IgG as antigens, and either the IgG fraction isolated from a goat antiserum to human immunoglobulin G, or goat antiserum to human light chain, as antibody.

Reaction with Anti-Human IgG Factor.—Binding capacity to antiglobulin factors was determined by inhibition of the latex fixation. The latex fixation test was carried out according to Singer et al. (33). Solutions of the native or treated IgG were mixed with an equal volume of a serum from a patient with rheumatoid arthritis and maintained 1 hr at room temperature. To 0.2 ml samples of serial dilutions of these mixtures, 0.2 ml of IgG-coated latex was added. After 90 min incubation at 56°C the mixtures were kept overnight at 4°C, centrifuged for 5 min at 2000 rpm and graded macroscopically.

Genetic Factors.—Four genetic factors, Gm(1), Gm(2), Gm(4), and Gm(12) (nomenclature recommended in reference 34), were determined on both native and treated IgG preparations by the method of Harboe (35). For each Gm factor determination, one drop of the sample to be tested was mixed on a microscopic slide with one drop of the respective anti-Gm serum and one drop of a 0.5% suspension of anti-Rh–coated erythrocytes. After 5–10 min at room temperature, the agglutination was read macroscopically. The specific anti-Gm reagents were obtained from Dr. Ropartz, of the Centre Departamental de Transfusion Sanguine et de Genetique Humaine, Rouen, France, whereas the specific anti-Rh sera were purchased from Biotest, Frankfurt/Main, Germany.

RESULTS

Cleavage of Human IgG by Cyanogen Bromide

Treatment of human IgG with cyanogen bromide under the same conditions as described for rabbit IgG (13), namely in 0.3 M HCl, led to almost complete precipitation of the protein. In control experiments (in the absence of CNBr) similar precipitation occurred, indicating that this phenomenon was due to

TABLE I

*Amino Acid Composition of Human IgG and Products of CNBr Digestion**

Amino acid residue	Human IgG‡	IgG from pooled normal serum	CNBr digest	F(ab″)₂
Lys	82	79	80	55
His	27	23	24	16
Arg	39	40	40	27
Asp	102	102	105	72
Thr§	105	102	105	79
Ser§	157	144	144	113
Glu	130	128	129	92
Pro	98	98	97	75
Gly	89	89	89	67
Ala	70	71	74	54
Val	108	117	121	82
Met	11	11.8	5.8	4
ILeu	29	30	30	21
Leu	99	99	96	73
Tyr	54	55	51	41
Phe	42	46	48	32

* The results were calculated from the analysis after hydrolysis for 22 hr, assuming the molecular weight of intact IgG to be 150,000, and that of $F(ab'')_2$ to be 100,000. The values in the table denote number of residues per molecule.

‡ Data from Crumpton and Wilkinson (36).

§ The values were not corrected for loss during hydrolysis.

the acidic conditions of the reaction. Milder acidic medium was, therefore, used for the cleavage of human IgG with CNBr, than was the case for rabbit IgG. No precipitation occurred and yet successful partial cleavage of human IgG was obtained upon treatment for 4 hr in 0.07 M CNBr–0.05 M HCl. Amino acid analysis of the lyophilized reaction mixture, as compared to the native IgG (Table I), demonstrated that half of the methionine residues in the original molecule had disappeared, while no change occurred in the contents of any other amino acid.

Attempts to remove the CNBr by dialysis against various buffers, ranging from 0.05 M acetate buffer, pH 4.5 to 0.1 M barbital buffer, pH 8.6, resulted

invariably in precipitation of the protein. Upon neutralization by one step addition of 1 M NaOH to pH 8, followed by dialysis against 0.5 M NaCl–0.02 M tris buffer, pH 8.2, the product remained in solution. However, the presence of large amounts of aggregates in such preparations, accompanying a product sedimenting with 5.8S, was demonstrated both by ultracentrifugal analysis (Fig. 1, D) and by measuring the ratio of their absorbancies at wavelengths 250 and 280 mμ; this ratio was 1.17, as compared to 2.55 for native IgG. The formation of these aggregates could not be avoided by addition of 0.4 M sodium decyl sulfate to the reaction mixture prior to the dialysis (Fig. 1, E), nor could they be eliminated by gel filtration on Sephadex G-100 or G-200 in 0.5 M NaCl–0.02 M tris buffer, pH 8.2, (Fig. 1, F) or in 1 M acetic acid (Fig. 1, C).

The method successfully used for the removal of lower molecular weight peptides as well as most of the CNBr from the original acidic reaction mixture was gel filtration on Sephadex G-100 in 0.05 M HCl. The elution pattern of a typical gel filtration is shown in Fig. 2; the main fraction recovered from the column was about 70% of the digestion mixture applied. Lyophilization of this product resulted in the removal of HCl and of the remainder CNBr. The water-soluble product obtained, migrated in the ultracentrifuge after exhaustive dialysis against 0.03 M NaCl (pH 5.8) as one main peak, with $s_{20,w}$ = 4.5S (Fig. 1, G). Native IgG migrated in 0.03 M NaCl with $s_{20,w}$ = 5.6S. The product with $s_{20,w}$ = 4.5S, to be denoted $F(ab'')_2$, had a ratio of absorbancy at 250 and 280 mμ of 2.25.

Physical and Chemical Characterization of $F(ab'')_2$

Solubility.—The $F(ab'')_2$, obtained by lyophilization was readily soluble in distilled water, yielding a solution of pH 2. It remained soluble after dialysis against distilled water at pH 5.8 or against 0.03 M NaCl, but precipitated upon dialysis against 0.15 M NaCl, against 0.01 M tris buffer, pH 7.3, or 0.1 M barbital buffer, pH 8.6. Dialysis against the same barbital buffer after previous raising of the pH with 1 M NaOH to pH 9 did not result in precipitation.

Starch Gel Electrophoresis.—As shown in Fig. 3, the $F(ab'')_2$ preparation obtained after the reaction of IgG with CNBr had a different electrophoretic pattern from that of native human IgG. The main fragment obtained had a higher mobility than that of the native protein and it was accompanied by a few fast migrating components. In contrast, IgG that was exposed to HCl in the absence of CNBr, did not show the presence of any components migrating faster than the IgG. These results indicate that by treatment with CNBr the molecule was cleaved in several places. Both the HCl control and the CNBr-treated IgG contained several slow components, which probably represent aggregates. The CNBr-produced fragment, after exposure to pH 9, shows more aggregated material than at pH 2.

Free Boundary Electrophoresis.—In 0.1 M barbital buffer, pH 8.6, $F(ab'')_2$

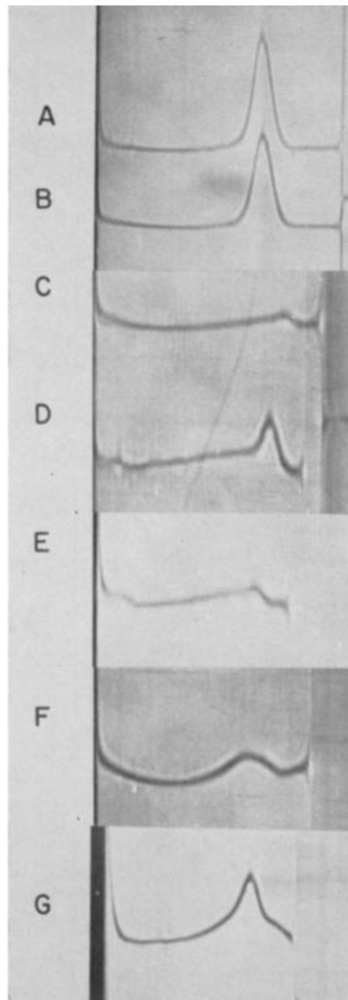

Fig. 1. Photograph of the Schlieren patterns taken in the Spinco model E ultracentrifuge after 32 min at 59,780 rpm. Sedimentation is from right to left. A, native IgG, and B, native antistreptolysin IgG, were run in 0.15 M NaCl–0.02 M phosphate buffer, pH 6.3; C, CNBr-treated IgG, fractionated on Sephadex G-100 in 1 M acetic acid and run in the same solvent; D, CNBr-treated IgG, fractionated on Sephadex G-100; and E, CNBr-treated IgG, fractionated on Sephadex G-200 in 0.5 M NaCl–0.02 M tris buffer pH 8.2, and run in the same solvent; F, CNBr-treated IgG, neutralized in presence of 0.1 M sodium decyl sulfate, fractionated, and run as in D. G, CNBr-treated IgG, fractionated on Sephadex G-100 in 0.05 M HCl and run in 0.03 M NaCl ($F(ab'')_2$).

appeared as a homogeneous material with a mobility of $\mu = 1.25 \times 10^{-5}$ cm²/v sec, as compared to 1.1×10^{-5} cm²/v sec of the IgG.

Amino Acid Analysis.—The amino acid analysis of $F(ab'')_2$ is given in Table I. The fragment still contained four methionine residues per a molecular weight of 100,000, whereas in the original CNBr-cleavage product there were

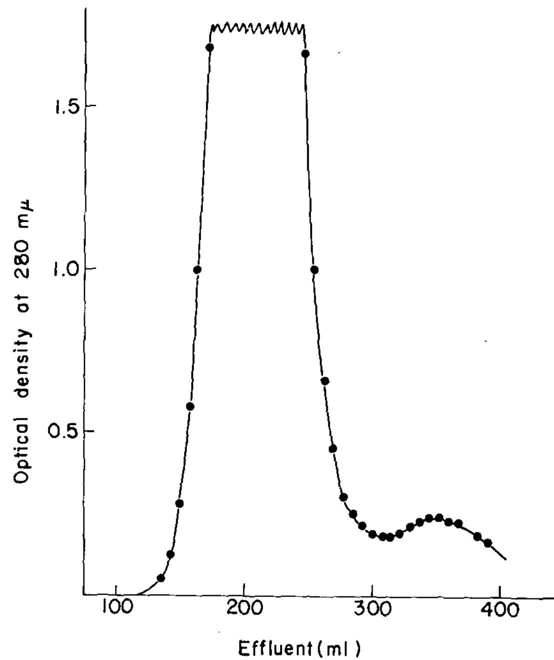

FIG. 2. Gel filtration pattern of the CNBr-digest of human IgG on Sephadex G-100 column (2.5×150 cm). The column was developed with 0.05 M HCl.

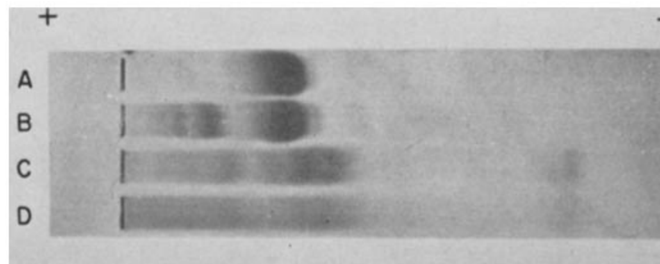

FIG. 3. Electrophoresis in 8 M urea-formic acid starch gel, pH 3.5. A, native IgG; B, IgG treated in 0.05 M HCl for 20 hr (control); C, aqueous solution at pH 5.8 of CNBr-treated IgG, previously fractionated on Sephadex G-100 in 0.05 M HCl and lyophilized ($F(ab'')_2$); D, same as C, in 0.1 M barbital buffer, pH 8.6.

six methionine residues per molecular weight of 150,000. Thus, all the methionine residues which were not converted into homoserine or homoserine lactone, are preserved in the F(ab″)$_2$.

Ultracentrifugal Analysis.—In Fig. 4 are shown the ultracentrifuge patterns in different media of F(ab″)$_2$, as compared to the original IgG. In aqueous

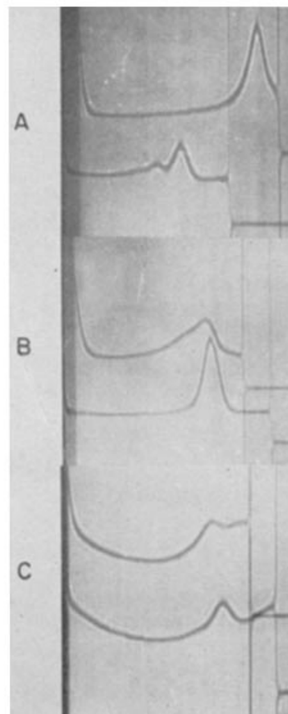

FIG. 4. Photograph of the Schlieren patterns taken in the Spinco model E ultracentrifuge after 32 min at 59,780 rpm. In each pattern the upper curve represents the CNBr-treated product and the lower curve represents the native IgG. A, aqueous solution, pH 2; B, aqueous solution, pH 5.8; C, 8 M urea.

solution at pH 2, F(ab″)$_2$ sedimented as an almost homogeneous peak of 2.6S, in comparison to the 5.5S of native IgG (Fig. 4, A). This acid pH caused a slight aggregation of the IgG. When tested in aqueous solution at pH 5.8 (Fig. 4, B), the native IgG had a sedimentation constant of 6.1S, whereas F(ab″)$_2$ showed a main peak (4.0S) with a shoulder (6.1S). In 8 M urea the respective sedimentation constants of the IgG and its cleavage products were 3.0S and 2.2S (Fig. 4, C). It seems, therefore, that under all experimental conditions tested the sedimentation rate of F(ab″)$_2$ is lower than that of the original protein, although absolute values are dependent on the solvent.

Biological Properties of F(ab″)₂

Antigenic Properties.—The treatment with CNBr under the mentioned specified conditions caused a marked decrease in the capacity of IgG to precipitate with specific goat antisera against the heavy chain. On the other hand the treated material retained its full precipitating capacity with goat anti–light chain sera. The reactions were followed both by quantitative precipitin reaction and by immunodiffusion in agar gel. In the quantitative precipitin reaction, $F(ab'')_2$ precipitated in the equivalence zone 27% of anti–heavy chain antibodies as compared to the native IgG, whereas it precipitated 102% of anti–light chain antibodies. The immunodiffusion experiments (Fig. 5) similarly indicated that, whereas native IgG or the HCl-treated control gave

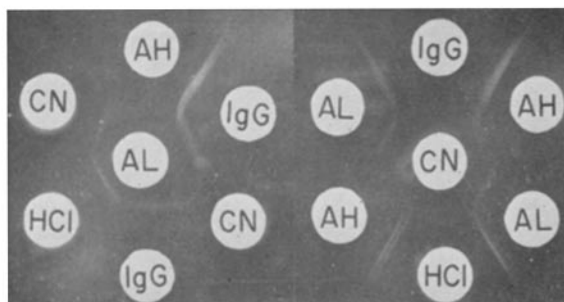

FIG. 5. Agar gel diffusion of the reaction of human IgG, before and after treatment with CNBr, with goat antisera to human heavy and light chains. AH, goat anti-human heavy chain; AL, goat anti-human light chain; IgG, native IgG; CN, CNBr-treated IgG; HCl, IgG-treated with HCl in the absence of CNBr.

two strong precipitation bands with anti–heavy chain, only a very faint band was observed with $F(ab'')_2$. In contrast, the reaction with anti–light chain demonstrated total identity between the three materials.

Residual Gm Determinants.—The various Gm factors were determined in two samples of IgG, namely, normal IgG and antistreptolysin IgG. The determinations were carried out with the native protein, with the HCl-treated control, and with the respective $F(ab'')_2$. As shown in Table II, the Gm(12) factor was completely abolished by the treatment with CNBr, whereas no change was caused by the treatment with HCl alone. On the other hand Gm(1) and Gm(4) determinants were unaffected by the action of CNBr. Gm(2) was not present on the samples of IgG studied.

Interaction with Human Antiglobulin Factors.—The reaction of IgG with the anti–γ-globulin factors, present in sera from patients with rheumatoid arthritis, has been demonstrated using inhibition of agglutination of latex coated with IgG. The inhibitory capacity of native IgG as compared to HCl-treated IgG and $F(ab'')_2$ is shown in Table III. Whereas the native IgG inhibited the ag-

glutination at a titer of 1/80, no inhibitory effect was caused by F(ab″)$_2$. It is pertinent to remark that the extent of inhibition caused by the HCl-treated IgG is even higher than that caused by the native material, probably due to partial denaturation and aggregation.

Complement-Binding Capacity.—Native IgG is capable of nonspecific binding of guinea pig complement, a property which is ascribed to possible aggregation via Fc portion of the molecule (37). This passive binding of complement is

TABLE II

The Effect of CNBr Treatment on the Genetic Markers of the Gm Type

Gm factors	Pooled human IgG			IgG with antistreptolysin activity		
	Native	HCl-control	F(ab″)$_2$	Native	HCl-control	F(ab″)$_2$
Gm(1)	+	+	+	+	+	+
Gm(12)	+	+	−	+	+	−
Gm(4)	+	+	+	+	+	+
Gm(2)	−	−	−	−	−	−

TABLE III

Inhibitory Capacity of Native IgG as Compared to Treated IgG on Interaction of IgG-Coated Latex with Antiglobulin Factors

Inhibition by	Dilution*							
	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
None	+	+	+	+	+	+	+	±
Native IgG	+	+	+	±	−	−	−	−
HCl-treated IgG	−	−	−	−	−	−	−	−
F(ab″)$_2$	+	+	+	+	+	+	±	−

* Dilutions of the rheumatoid arthritis serum alone and after mixing with different protein preparations.

decreased upon treatment with acid, but much more so by CNBr cleavage. Thus, as seen in Fig. 6, 4 mg of the native IgG caused almost complete inhibition of hemolysis, due to extensive binding of the complement, while the binding by F(ab″)$_2$ is almost negligible.

Reverse Passive Cutaneous Anaphylaxis.—In order to study the skin-binding capacity of IgG, either native, or CNBr-treated, we could not use the direct passive cutaneous anaphylaxis, since the pooled normal IgG did not possess any defined antibody activity. Recourse was made, therefore, to the reverse passive cutaneous anaphylaxis (32) where the IgG was injected intradermally as the antigen, while goat antiserum was injected intracardially as the antibody source. The results of this experiment with anti–heavy chain and anti–light

chain sera are shown in Table IV. The reaction with anti–heavy chain measures the skin binding but is dependent also on the residual antigenic activity of $F(ab'')_2$. On the other hand, the reaction with the anti–light chain is a direct indication of the skin-binding capacity, since the treated protein had retained

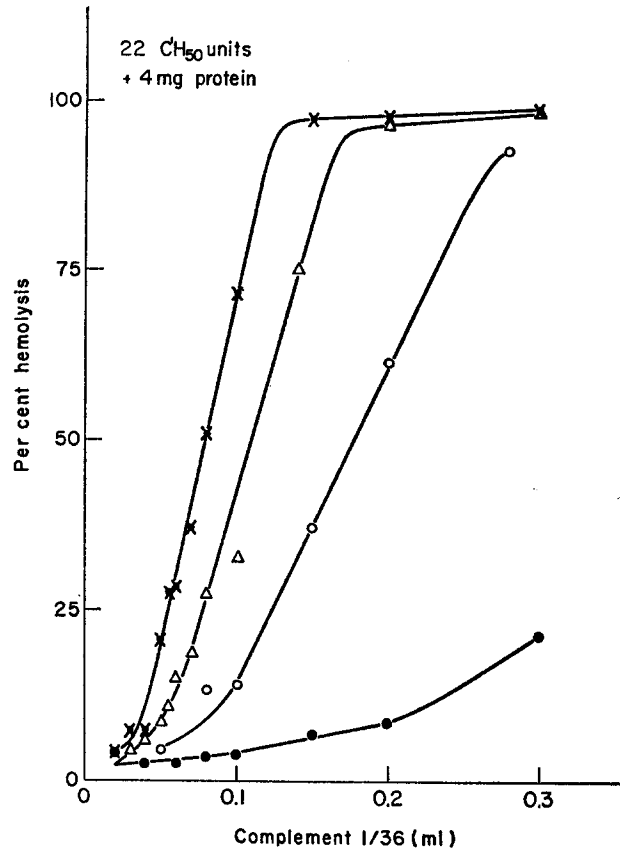

FIG. 6. Complement binding by native IgG (●), HCl-treated IgG (○), and CNBr-treated IgG (△). (×), the titration curve of the complement.

its full capacity to react with anti–light chain serum. The results show that while the native IgG gave a positive reaction with the antisera when injected at a level of 0.33 μg or less, $F(ab'')_2$ reacted with the anti–heavy chain serum only, at a level of 5 μg, and did not react at all with the anti–light chain serum.

Antibody Activity.—In order to investigate whether antibody activity of IgG is affected by treatment with CNBr, ues was made of IgG samples prepared from sera possessing specific activity.

Antistreptolysin Activity.—Sera of patients suffering from rheumatic fever

TABLE IV

Reverse Passive Cutaneous Anaphylaxis Reactions

Amount of protein injected intradermally	Diameter of lesion, *mm*			
	Challenge with native IgG		Challenge with $F(ab'')_2$	
	Anti-heavy chain	Anti-light chain	Anti-heavy chain	Anti-light chain
μg				
50	15	16	11	0
10	15	11	8	0
5	14	10	0	0
2.5	12	Not done	0	Not done
1	12	Trace	0	0
0.33	8	Not done	0	Not done
0.11	Trace	0	0	0
0.036	0	Not done	0	Not done

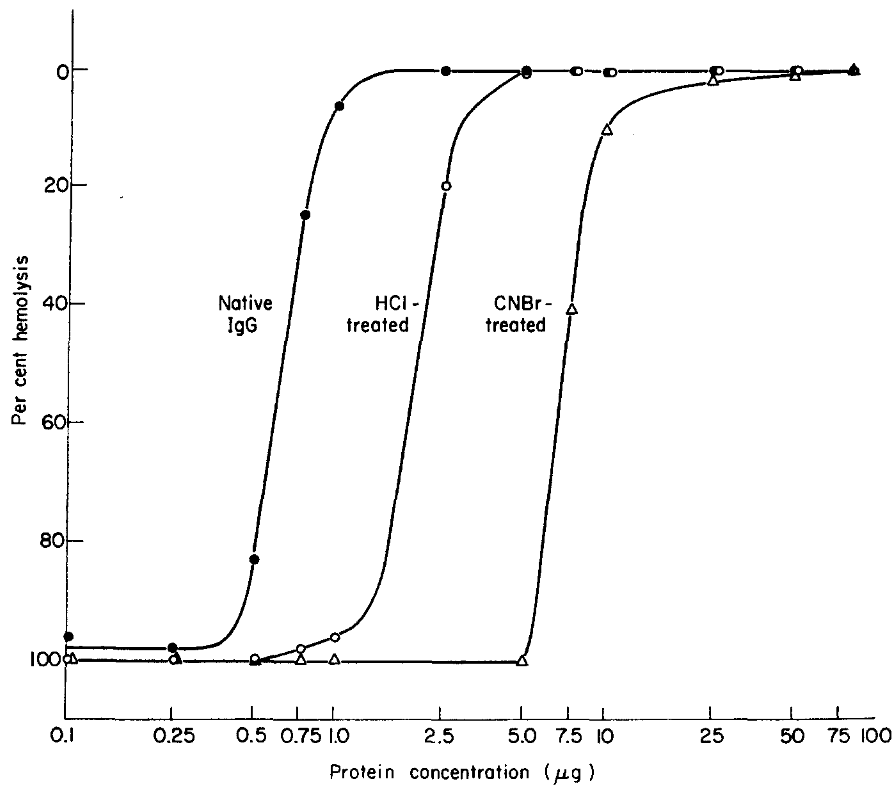

FIG. 7. Effect of CNBr-cleavage and HCl-treatment on antistreptolysin activity of human IgG.

are known for their capacity to react with streptolysin O (38). Since streptolysin O causes hemolysis of human red blood cells, its interaction with the specific antistreptolysin in such sera is manifested by the inhibition of hemolysis. Fig. 7 shows the extent of hemolysis which was caused by a constant amount of streptolysin after interaction with increasing quantities of native IgG, HCl-treated IgG, or $F(ab'')_2$. Although a certain decrease in antistreptolysin activity is observed after the CNBr or HCl treatment, the protein still retains most of its activity.

Isohemagglutinin Activity.—The IgG isoagglutinin prepared from a normal type O individual, as well as the $F(ab'')_2$ derived from it, were tested for hemagglutinating activity using erythrocytes of type A (Table V). The threshold concentration of IgG was 0.03 mg/ml and $F(ab'')_2$ gave a negative reaction at a concentration of 0.04 mg/ml; both gave a strongly positive reaction at 0.06 mg/ml, indicating that the capacity to agglutinate the appropriate red blood cells was not impaired by the treatment with CNBr.

TABLE V

Isohemagglutination Reaction with IgG and $(Fab'')_2$

Sample	Concentration, *mg/ml*						
	0.24	0.15	0.1	0.06	0.04	0.03	0.015
IgG	+++	+++	+++	++	±	±	−
$F(ab.)^2$	+++	+++	+++	++	−	−	−

DISCUSSION

The specific reaction of cyanogen bromide with polypeptide chains, leading to the selective cleavage of peptide bonds adjacent to the methionine residues, necessitates an acid medium. Conditions as drastic as 70% formic acid were used for quantitative cleavage of all the methionyl bonds in human as well as rabbit IgG (14–17). Such treatment results in complete loss of biological activity. On the other hand, treatment of rabbit IgG with CNBr in 0.3 M HCl, allowed the cleavage of approximately half of the methionyl bonds in the IgG molecule, yielding a biologically active 5S fragment (denoted $F(ab'')_2$ and several smaller peptides (13).

In the present study it was found that still milder conditions for cleavage with CNBr have to be used in the reaction with human IgG, in order to obtain a soluble fragment possessing biological activity. When the reaction with CNBr was allowed to proceed in 0.05 M HCl, cleavage indeed took place, as was demonstrated both by decrease in the methionine content of the hydrolysate, and by starch gel electrophoretic and ultracentrifugal analyses. Gel filtration in 0.05 M HCl removed most of the smaller fragments, and yielded a main frac-

tion which, at low pH, appeared as a homogenous peak in the ultracentrifuge (Fig. 4, A). Neutralization by several procedures resulted in formation of aggregates. Macroscopic precipitation of about 70% of the material occurred upon gradual raise of the pH, whereas adjustment of the solution to pH 8.6 in one step resulted in a material which, though soluble, showed the presence of aggregates. The aggregation is a process depending not only on the pH but on the ionic strength as well. Thus, at pH 5.8 and very low ionic strength, the material is mostly in the nonaggregated form (Fig. 1, G), while the extent of aggregation increases with an increase in the ionic strength.

The product of the cleavage of human IgG with CNBr, sedimenting with 4.5S in 0.03 M NaCl at pH 5.8, and denoted here $F(ab'')_2$, is analogous to the "5S-CNBr" product obtained upon cleavage of rabbit IgG with CNBr (13). The lower sedimentation coefficient is due to the changed conditions of the ultracentrifugation. Thus, intact human IgG sedimented under these conditions (0.03 M NaCl at pH 5.8) with 5.6S.

The pH profile of the aggregation of the CNBr–cleavage product is different from that of the intact IgG. The latter is known to aggregate at low pH, presumably via the Fc fragment, which forms by itself aggregates at low pH (39). Beside sedimentation in the ultracentrifuge, the presence of aggregates was followed also by comparison of absorbancies at 250 and 280 mμ. The measurements at 250 mμ are much stronger affected by aggregation than those at 280 mμ.

The $F(ab'')_2$ fragment obtained upon cleavage with CNBr and isolated by gel filtration (Fig. 2) represented approximatively 70% of the native IgG. It migrated as a homogeneous material in free boundary electrophoresis but, starch gel electrophoresis showed, in addition to the main fraction, small amounts of components with higher electrophoretic mobility. Thus gel filtration did not remove quantitatively the smaller cleavage products.

The main object of this investigation was to determine to what extent will the different biological properties of IgG be affected by cleavage with CNBr. The capacity to react with specific antigen remained practically unchanged, as was demonstrated by two independent systems. The samples of IgG used for these experiments were not deliberately prepared antisera, but individual sera having specific antibody activity. The isoagglutinin activity did not change significantly as the result of the action of CNBr (Table V), whereas only a partial decrease in antistreptolysin activity was observed (Fig. 7). The assay used for antistreptolysin activity in this study measures directly the interaction of antigen and antibody and does not require any auxiliary system. In a study performed with a similar divalent antibody fraction, obtained by treatment of human IgG with pepsin, and using the complement-fixation method, antistreptolysin activity could not be demonstrated, due to the inability of the system to bind complement (40).

In contrast to the antibody activity, a drastic change in the various characteristics related to the antigenic determinants of the IgG was observed. The reaction with specific goat antisera indicated that while the antigenic determinants on the light chain are present to the full extent on $F(ab'')_2$, those related to the heavy chain are only partially retained.

In the reverse passive cutaneous anaphylaxis, the reaction of $F(ab'')_2$ was almost negative when challenged either with goat anti–heavy chain or with anti–light chain sera. The lack of reaction with the anti–heavy chain could be interpreted as due either to the absence of the area of the IgG molecule responsible for fixing to skin, or to the loss of the antigenic determinants of the heavy chain of human IgG. On the other hand, the negative result with anti–light chain demonstrates that the action of CNBr indeed removed the area of the IgG molecule responsible for fixation to skin. The lesions obtained upon reacting $F(ab'')_2$ in high concentration with the anti–heavy chain serum were probably due to contamination with some intact IgG.

Since most of the antigenic determinants of the IgG are assumed to be on the Fc portion of the molecule (7), and the skin-binding capacity is attributed to the Fc portion as well (9), it can be concluded that, in the human IgG, as in the case of rabbit IgG (13), the CNBr cleavage caused a preferential destruction of that portion of the molecule with which Fc is associated. This assumption is in accord with the finding that $F(ab'')_2$ is not capable of reacting with antiglobulin factors, in contrast to intact IgG (41).

Efforts to localize the genetic markers, showed that the isolated $F(ab'')_2$ fragment is lacking Gm(12), but still contains both Gm(1) and Gm(4). The presence of Gm(4) and the absence of Gm(12) are in agreement with previous reports (42), drawing conclusions about the location of various genetic markers on the IgG molecule from studies of the enzymatic degradation products. On the other hand, the presence of Gm(1) on $F(ab'')_2$ may be due to one of two reasons. One possibility is that this marker is located on the portion of the heavy chain between the locus of cleavage with papain and the locus of cleavage with CNBr, whereas the Gm(12) is located closer to the COOH terminus of the heavy chain. A corollary of this assumption is that the CNBr cleavage of the heavy chain occurs between the Gm(12) and Gm(1) markers. The other possibility, which cannot yet be excluded, is that the Gm(1) marker is on a peptide which was cleaved away from $F(ab'')_2$ by CNBr but was not completely removed thereafter.

Another biological property investigated is the anticomplementary activity. IgG fractionated from serum is capable of nonspecifical binding of complement (43). Evidence has been presented (44, 45) that this effect is increased by artificial aggregation of the IgG, and therefore it was assumed that presence of aggregates is the cause of this complement fixation. Attempts to reduce these effects have thus been concentrated either on efforts to remove such aggre-

gates by high speed centrifugation, or by the use of low pH as a disaggregating means (43). Enzymatic fragmentation of the IgG by papain led to fragments which were incapable of binding complement (39). On the other hand, aggregation experiments demonstrated that Fc alone could form aggregates fixing complement. It seems, thus, that the complement-fixing capacity is inherent in the Fc portion of the IgG molecule, and is not due to aggregation per se. The results presented in this study sustain this hypothesis by demonstrating that the CNBr-digest product of IgG, in which Fc is presumably absent, has lost the anticomplementary activity of the original IgG, although it is not free of aggregates under the specified experimental conditions. The results also demonstrate that, although acid treatment alone causes a marked loss of complement-binding capacity, the effect of CNBr cleavage is more pronounced (Fig. 6).

Investigations carried out using CNBr for cleavage of proteins, were concerned mainly with structural and amino acid sequence studies. The purpose of the present study was to obtain a fragment which will retain the antibody function of the starting immunoglobulin. The mere fact that the isohemagglutinating activity was preserved after CNBr cleavage of IgG containing anti-A activity indicates that the CNBr-produced $F(ab'')_2$ fragment is indeed divalent. Thus it may be deduced that, in resemblance to the rabbit IgG (13), human IgG may be cleaved by CNBr to yield a product similar to the one obtained by pepsin degradation.

It has been suggested that human IgG degraded with pepsin may be suitable for intravenous administration (40). The observation presented here, that the main product of CNBr cleavage of human IgG under the conditions described, kept its antibody activity, whereas it lost most of its hetero- and isoantigenic determinants, as well as its capacity to bind complement and fix to skin, suggests similarly the advisability of testing CNBr-cleaved human IgG for possible therapeutic uses. The advantage of using CNBr for cleavage of IgG is in its defined specificity and in the ease of removal of the reagent.

SUMMARY

Treatment of human IgG with cyanogen bromide in 0.05 M HCl under specified conditions resulted in the cleavage of about half of its methionyl peptide bonds. A major fragment of about 5S was isolated from the reaction mixture by gel filtration in quantitative yield. The CNBr fragment reacted fully with goat antiserum against human light chain, but its reaction with anti–heavy chain was markedly decreased. The treatment with CNBr caused a drastic decrease in the following biological activities of IgG: complement fixing, skin binding, reaction with antiglobulin factors, and reaction with specific anti-Gm(12) serum. On the other hand, the reaction with serum of anti-Gm(1) or anti-Gm(4) specificity was not impaired and antibody activity, namely

antistreptolysin and isohemagglutinin, was retained after the treatment with CNBr.

It is concluded that the CNBr cleaves preferentially the methionyl bonds in the Fc portion of IgG, and that the major fragment obtained, denoted F(ab″)$_2$, has still the combining properties of a divalent antibody. The possible therapeutic uses of F(ab″)$_2$ are discussed.

The assistance of Mr. Ariel Lustig with the ultracentrifugal analyses, and of Miss Hanna Levinhar with the determination of genetic markers, is gratefully acknowledged.

BIBLIOGRAPHY

1. Porter, R. R., and E. M. Press. 1964. Antibodies and γ-globulin. *Ann. Rev. Biochem.* **31**:625.
2. Franklin, E. C. 1960. Structural units of human 7S gamma globulin. *J. Clin. Invest.* **39**:1933.
3. Edelman, G. M., J. F. Heremans, M. T. Heremans, and H. G. Kunkel. 1960. Immunological studies of human γ-globulin. *J. Exptl. Med.* **112**:203.
4. Deutsch, H. F., E. R. Stiehm, and J. I. Morton. 1961. Action of papain on human serum globulins. *J. Biol. Chem.* **236**:2216.
5. 1964. Nomenclature for human immunoglobulins. *Bull. World Health Organ.* **30**:447.
6. Nisonoff, A., F. C. Wissler, and L. N. Lipman. 1960. Properties of the major component of a peptic digest of rabbit antibody. *Science.* **132**:1770.
7. Edelman, G. M., and M. D. Poulik. 1961. Studies on structural units of the γ-globulins. *J. Exptl. Med.* **113**:861.
8. Deutsch, H. F., N. O. Thorpe, and H. H. Fudenberg. 1963. Some biological activities of papain-produced subunits of human 7S gamma globulins. *Immunology.* **6**:539.
9. Ishizaka, K., T. Ishizaka, and T. Sugahara. 1962. Biological activity of soluble antigen-antibody complexes. VII. Role of an antibody fragment in the induction of biological activities. *J. Immunol.* **88**:690.
10. Ovary, Z. 1958. Immediate reaction in the skin of experimental animals provoked by antibody-antigen interaction. *Progr. Allergy.* **5**:459.
11. Harboe, M., C. K. Osterland, and H. G. Kunkel. 1962. Location of two genetic factors to different areas of γ-globulin molecules. *Science.* **136**:979.
12. Gross, E., and B. Witkop. 1962. Nonenzymatic cleavage of peptide bonds: The methionine residues in bovine pancreatic ribonuclease. *J. Biol. Chem.* **237**:1856.
13. Cahnmann, H. J., R. Arnon, and M. Sela. 1966. Isolation and characterization of active fragments obtained by cleavage of immunoglobulin G with cyanogen bromide. *J. Biol. Chem.* **241**:3247.
14. Givol, D., and R. R. Porter. 1965. The C-terminal peptide of the heavy chain of the rabbit immunoglobulin IgG. *Biochem. J.* **97**:32C.
15. Press, E. M., D. Givol, P. J. Piggot, R. R. Porter, and J. M. Wilkinson. 1966. The chemical structure of the heavy chains of rabbit and human immunoglobulin G (IgG). *Proc. Roy. Soc. (London) Ser. B.* **166**:150.
16. Press, E. M., P. J. Piggot, and R. R. Porter. 1966. The N-terminal and C-terminal

amino acid sequences of the heavy chain from a pathological human immunoglobulin IgG. *Biochem. J.* **99:**356.

17. Sjöquist, J. 1966. Degradation of human γG-globulin with cyanogen bromide. *J. Mol. Biol.* **20:**537.
18. Weir, R. C., R. R. Porter, and D. Givol. 1966. Comparison of the C-terminal amino acid sequence of two horse immunoglobulins IgG and IgG(T). *Nature.* **212:**205.
19. Cahnmann, H. J., R. Arnon, and M. Sela. 1965. Active fragments obtained by cleavage of rabbit antibody with cyanogen bromide. *J. Biol. Chem.* **240:**P.C. 2763.
20. Koshland, M. E. 1966. Primary structure of immunoglobulins and its relationship to antibody specificity. *J. Cellular Comp. Physiol.* **67:**(Suppl. 1): 33.
21. Levy, H. B., and H. A. Sober. 1960. A simple chromatographic method for preparation of gamma globulin. *Proc. Soc. Exptl. Biol. Med.* **103:**250.
22. Schultze, H. E., and J. F. Heremans. 1966. Molecular Biology of Human Proteins. Elsevier Publishing Company, Amsterdam. **1.**
23. Spackman, D. H., W. H. Stein, and S. Moore. 1958. Automatic recording apparatus for use in the chromatography of amino acids. *Anal. Chem.* **30:**1190.
24. Edelman, G. M., and M. D. Poulik. 1961. Studies on structural units of the γ-globulins. *J. Exptl. Med.* **113:**861.
25. Longsworth, L. G. 1953. Improved electrophoresis apparatus for small volumes. *Anal. Chem.* **25:** 1074.
26. Fleischman, J. B., R. R. Porter, and E. M. Press. 1963. The arrangement of peptide chains in γ-globulin. *Biochem. J.* **88:**220.
27. Ouchterlony, Ö. 1953. Antigen-antibody reactions in gels. IV. Types of reactions in coordinated systems of diffusion. *Acta Pathol. Microbiol. Scand.* **32:**231.
28. Mayer, M. M., A. G. Osler, O. G. Bier, and M. Heidelberger. 1948. Quantitative studies of complement fixation. I. A method. *J. Immunol.* **59:**195.
29. Mayer, M. M., A. G. Osler, O. G. Bier, and M. Heidelberger. 1946. The activating effects of magnesium and other cations on the hemolytic function of complement. *J. Exptl. Med.* **84:**535.
30. Rantz, L., and E. Randall. 1945. A modification of the technic for determination of the antistreptolysin titer. *Proc. Soc. Exptl. Biol. Med.* **59:**22.
31. Kabat, E. A., and M. M. Mayer. 1961. Experimental Immunology. Charles C Thomas, Springfield, Ill. p. 149.
32. Ovary, Z. 1960. Reverse passive cutaneous anaphylaxis in the guinea pig with horse, sheep, or hen antibodies. *Immunology.* **3:**19.
33. Singer, J. M., G. Altmann, A. Goldenberg, and C. M. Plotz. 1960. The mechanism of particulate carrier reactions with rheumatoid sera. II. Sensitizing capacity of various human gamma globulins for latex particles. *Arthritis Rheumat.* **3:**515.
34. 1965. Notation for genetic factors of human immunoglobulins. *Bull. World Health Organ.* **33:**No. 5.
35. Harboe, M. 1959. Quantitative studies on the hemagglutination inhibition reaction for determination of the Gm types. *Acta Pathol. Microbiol. Scand.* **47:**199.
36. Crumpton, M. J., and J. H. Wilkinson. 1963. Amino acid composition of human and rabbit γ-globulins and of the fragments produced by reduction. *Biochem. J.* **88:**228.

37. Cremer, N. E., J. L. Riggs, E. M. Leunette, and F. W. Jensen. 1965. Nonspecific binding of complement by digestion fragments from antiviral gamma globulin. *Science.* **149**:84.
38. Vorlander, K. O. 1961. Immunologische Untersuchungen bei entzundlichen-rheumatischen Erkrankungen. *In* Immunopathologie in Klinik und Forschung. P. Miescher, and K. O. Vorlander, editors. Thieme Verlag., Stuttgart, Germany.
39. Thorpe, N. O., and H. F. Deutsch. 1966. Studies on papain-produced subunits of human γ G-globulins. I. Physicochemical and immunochemical properties of γ-G globulin Fc fragments. *Immunochemistry.* **3**:317.
40. Schultze, H. E., and G. Schwick. 1962. Uber neue Möglichkeiten intravenöser Gammaglobulin-Applikation. *Deut. Med. Wochschr.* **87**:1643.
41. Goodman, J. W. 1961. Reaction of rheumatoid sera with fragments of papain-digest rabbit gamma globulin. *Proc. Soc. Exptl. Biol. Med.* **106**:822.
42. Mårtensson, L. 1966. Genes and immunoglobulins. *Vox Sanguinis.* **11**:521.
43. Barandun, S., P. Kistler, F. Jeunet, and H. Isliker. 1962. Intravenous administration of human γ-globulin. *Vox Sanguinis.* **7**:157.
44. Ishizaka, T., and K. Ishizaka. 1959. Biological activities of aggregated gamma globulin. I. Skin reactive and complement fixing properties of heat denatured gamma globulin. *Proc. Soc. Exptl. Biol. Med.* **101**:845.
45. Ishizaka, K., and T. Ishizaka. 1960. Biologic activity of aggregated γ-globulin. II. A study of various methods for aggregation and species differences. *J. Immunol.* **85**:163.