Complement Fixing Site of Human IgG1

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Abstract □ A number of chemical modifications were made to purified human IgG1. The effects of these modifications on the complement activity of the immunoglobulin were studied using aggregation on latex and measuring the consumption of the complement by CH_{50}-quantitation. Tryptophan and tyrosine are implicated in the complement fixing site of this human immunoglobulin, and an arginine moiety probably provides a binding site for the complement.

Keyphrases □ Complement fixing—human IgG1, effect of chemical modifications on active site □ Immunoglobulins—human IgG1, effect of chemical modifications on complement activity □ Amino acids—residues of human IgG1 involved in complement fixing defined

It has been shown that modification of a few tryptophan residues of rabbit immunoglobulin G with 2-hydroxy-5-nitrobenzyl bromide reduces the anticomplementary activity of this protein (1, 2). This observation prompted interest in defining some of the amino acid residues of human immunoglobulin G1 (IgG1[1]) involved in its complement fixing ability. For this purpose, studies were conducted on the change in anticomplementary activity by chemical modification of human IgG1.

EXPERIMENTAL

Preparation of Human IgG1—The plasma[2] containing IgG1 was isolated from a patient with multiple myeloma. Sufficient ϵ-aminocaproic acid was added to the plasma to make the final concentration of the amino acid 1×10^3 *M*. For every 100 ml of plasma, 1 ml of 25% $CaCl_2$ and 1 ml of 10% NaN_3 were added. The material was allowed to clot overnight at 4°, the clot was then removed, and ammonium sulfate (50%) precipitation of the serum was performed.

The precipitate was dissolved in distilled water and reprecipitated with ammonium sulfate (33%). This latter precipitation was repeated. Then the precipitate was dissolved in distilled water and dialyzed against tris(hydroxymethyl)aminomethane buffer, 0.005 *M*, pH 8.0. This solution was concentrated[3] and then chromatographed on a column (44 × 2.5 cm) of cellulose[4] in the same buffer. Elution of the column with 0.015 *M* tris(hydroxymethyl)aminomethane, pH 8.0, produced human IgG1.

The purity of the product was indicated by Ouchterlony double diffusion in agar gels using goat anti-human serum, from which only a single precipitin band was obtained. Similarly, only a single band was obtained upon polyacrylamide electrophoresis of the sample of human IgG1.

Modifications of Human IgG1—*2-Hydroxy-5-nitrobenzyl Bromide (I)*—The reaction with I was performed in a similar manner to that described by Allen and Isliker (1, 2). To 4 ml of a 2.7% solution of human IgG1 in 0.1 *M* sodium acetate buffer, pH 4.5, was added 0.2 ml of a 0.33 *M* dioxane solution of I. The mixture was stirred for 1 hr at room temperature, centrifuged, and then dialyzed for 24 hr against 4 × 250 ml of 0.1 *M* sodium acetate. The number of moles of I bound per mole of IgG1 was calculated by determining the absorbance at 410 nm (E_{410} = 18,000 M^{-1} cm^{-1}, pH >12.0) (6) for the modified IgG1 and at 280 nm ($E^{1\%}_{1cm}$ = 13.6, pH 7.0) for IgG1. A molecular weight of 150,000 was used for human IgG1.

5-(Dimethylamino)-1-naphthalenesulfonyl Chloride (II)—The reaction with II was performed in a similar manner to that previously reported (7, 8). Typically, 2.50 ml of a 7.4×10^{-3} *M* II solution in acetone was added to 8.75 ml of a 5.4% solution of human IgG1 dissolved in 0.1 *M* $NaHCO_3$, pH 8.0. The mixture was stirred for 2 hr at 4°. The ratio of II to protein was 6:1. The reaction mixture was dialyzed for 24 hr against 4 × 250 ml of 0.05 *M* tris(hydroxymethyl)aminomethane–0.15 *M* NaCl, pH 8.0. Then the solution was centrifuged, and the number of moles of the 5-(dimethylamino)-1-naphthalenesulfonyl moiety bound per mole of IgG1 was calculated by determining the absorbance at 280 nm ($E^{1\%}_{1cm}$ = 13.6, pH 7.0) for IgG1 and at 335 nm ($E_{335} = 4.4 \times 10^6\ M^{-1}\ cm^{-1}$) for bound IgG1. A molecular weight of 150,000 was assumed for human IgG1.

Tetranitromethane (III)—The method of Atassi and Habeeb (9) was used for the nitration of human IgG1. To a solution of 1.8 μmoles of human IgG1 in 5 ml of 0.05 *M* tris(hydroxymethyl)aminomethane–1 *M* NaCl, pH 8.0, was added 21.4 μmoles of III in 75 μl of ethanol. The mixture was stirred at room temperature for 2 hr and then dialyzed for 24 hr against 4 × 250 ml of barbital-buffered saline, pH 7.4, containing 0.15 m*M* calcium and 1.0 m*M* magnesium ions (10). The number of moles of nitrotyrosine associated with each mole of IgG1 was calculated by determining the absorbance at 280 nm ($E^{1\%}_{1cm}$ = 13.6, pH 7.0) for IgG1 and at 428 nm ($E = 3800\ M^{-1}\ cm^{-1}$) (11) for nitro IgG1. Amino acid analysis of the modified IgG1 confirmed this result.

2,3-Butanedione (IV)—This modification of human IgG1 was done using the method of Grossberg and Pressman (12). To 2 ml of a 5.4% solution of human IgG1 in 0.001 *M* phosphate buffer, pH 7.5, was added 2 ml of IV reagent (optical density = 0.5/ml), and the pH of the reaction mixture was adjusted to 8.6. After 6 hr at room temperature, the mixture was centrifuged and dialyzed for 24 hr against 4 × 250 ml of barbital-buffered saline, pH 7.4, containing 0.15 m*M* calcium and 1.0 m*M* magnesium ions (10). Comparison of the amino acid analysis of acid hydrolysates of IgG1 and modified IgG1 showed that half of the arginine residues of the immunoglobulin had been modified.

Protein Absorption on Latex Particles—To 0.25-ml aliquots of an aqueous 1.5% latex suspension (particle size 0.81 μm) was added 1 ml of barbital-buffered saline, pH 7.4, containing 0.15 m*M* calcium and 1.0 m*M* magnesium ions (8). To each sample was added 20 μg of the pertinent human IgG1 or its modifications, and the suspension was gently inverted through 180° every 5 sec to ensure complete mixing. Complete adsorption of the protein to the latex was accomplished by incubation for 16 hr at 4°.

Hemolytic Complement Fixation Assay—The method employed for the quantitative fixation test was based on one described previously (10). All operations were performed in barbital-buffered saline, pH 7.4, containing optimal concentrations of calcium and magnesium ions. The protein–latex suspensions were centrifuged, and to each pellet was added 1.2 ml of human complement containing 20 CH_{50} units/ml. Each pellet was suspended in the complement and incubated for 2 hr at 37°. The suspensions were inverted every 15 min to ensure reaction.

After this incubation, the suspensions were centrifuged and 1 ml of the supernate from each reaction mixture was removed. These supernates were assayed for residual complement activity. Each supernate was diluted in ratios of 1:7, 1:10, and 1:15 with barbital-buffered saline containing 0.15 m*M* calcium and 1.0 m*M* magnesium ions. To 1-ml aliquots of each dilution were added 5×10^8 sensitized sheep erythrocytes, and

Table I—Anticomplementary Activity of Modified Human IgG1

Protein[a]	Amount of Modification[a]	Number of CH_{50} Units Removed per Microgram of Protein	Decrease in Anticomplementary Activity, %
Human IgG1	None	1	0
II-Bound IgG1	4.5	0.65	35
I-Bound IgG1	6.6	0.41	59
Nitro IgG1	7.3	0.28	72
IV-Modified IgG1	16.0	0.23	77

[a] The amount of protein used was 20 μg adsorbed to 3.75 mg of latex. [b] Moles of chemical modifier per mole of immunoglobulin.

[1] Nomenclature recommended by International Union of Immunological Societies (3–5).

[2] Courtesy of Dr. W. T. Hammack, Veterans Administration Hospital, Birmingham, Ala.

[3] Diaflo-filtration using a PM-30 filter.

[4] DE-52.

Table II—Comparison of Amino Acid Sequences of Various Anticomplementary Immunoglobulins

Immunoglobulin C_H2 Domain	Amino Acid Residues	Sequence
Human IgG1 (Ref. 13)	271–282	Pro-Gln-Val-Lys-Phe-Asn-Trp-Tyr-Val-Asp-Val-Gln
Guinea pig IgG2 (Ref. 14)	271–282	Pro-Glu-Val-Gln-Phe-Thr-Trp-Phe-Val-Asp-Lys-Pro
Rabbit IgG (Ref. 15)	271–282	Pro-Glu-Val-Glx-Phe-Thr-Trp-Ile-Asx-Glu-Gln-Val
Murine IgG2a (Ref. 16)	271–282	Pro-Asp-Val-Gln-Ile-Ser-Trp-Phe-Val-Asp-Val-Glu

the volumes were made up to 7.5 ml with the barbital buffer. These suspensions were incubated for 1 hr at 37° and then centrifuged.

The optical density of each supernate was obtained at 541 nm. The following controls were run concurrently with the latex–protein samples: no latex–protein, latex only, sensitized sheep erythrocytes without complement, complement only, and total lysis of sensitized sheep erythrocytes.

RESULTS

The 2,3-butanedione reagent (12) modified 16 moles of arginine/mole of human IgG1. The amino acid analysis indicated no reaction with the lysine residues. Each mole of human IgG1 bound 4.5 moles of 5-(dimethylamino)-1-naphthalenesulfonyl moiety. Nitration, using tetranitromethane, of human IgG1 indicated 7.3 moles of nitrotyrosine bound/mole of immunoglobulin. Alkylation of the tryptophan residues with 2-hydroxy-5-nitrobenzyl bromide showed that 6.6 moles of this alkylating agent was bound per mole of IgG1.

To quantitate the complement fixing abilities of the human IgG1 and its various modifications, it was necessary to aggregate the various proteins on latex particles. The standard used was 20 μg of human IgG1 aggregated to 3.75 mg of latex particles. At this ratio, all immunoglobulin was adsorbed. This amount of IgG1 routinely removed all of the measurable complement from human serum. Of the controls run concurrently with the samples, the latex by itself removed 5–10% of the available complement. This reduction of complement for consumption by the immunoglobulins has been incorporated in the results shown in Table I.

By using these standard conditions, it was possible to compare the complement fixing abilities of human IgG1 with those of the various modifications of the immunoglobulin. Compared to the native immunoglobulin, the complement fixing activity of the alkylated material was reduced by 59%. Binding of human IgG1 to II was not as effective, causing a decrease of 35% in the complement fixing ability of the modified immunoglobulin. Nitration of human IgG1 lowered the anticomplementary activity by 72%. This finding suggests that a tyrosine residue may be implicated as a moiety in the complement fixing area of the immunoglobulin. The reaction of IgG1 with the 2,3-butanedione reagent (12) gave the greatest decrease, 77%, in the complement fixing activity of the immunoglobulin. However, amino acid analysis of this material indicated that only the arginine residues were affected by this reagent.

DISCUSSION

Allen and Isliker (1, 2) showed that modification of rabbit IgG with 2-hydroxy-5-nitrobenzyl bromide reduced the complement fixing ability of the immunoglobulin. It was suggested that the moiety modified by this alkylating agent was a tryptophan residue located in the Fc portion (3–5) of the immunoglobulin close to the hinge region (3–5). Furthermore, the tryptophan at position 277 within the first domain of the Fc portion of rabbit immunoglobulin appears to be involved in complement fixation (1).

With human IgG1, a similar change in anticomplementary activity was observed when tryptophan was alkylated. Nitration of the tyrosine residues also affected the anticomplementary activity of the modified immunoglobulin. Comparison of the sequences of immunoglobulins that possess anticomplementary activity is shown in Table II. There is a great similarity of amino acid residues around the tryptophan moiety at position 277. The close proximity of the aromatic residues, phenylalanine and tyrosine, is also evident. Possibly nitration of human IgG1 with tetranitromethane modified one of these residues.

The present results also implicate an arginine residue playing a role in complement consumption. However, the sequences shown in Table II do not include this amino acid residue. It has been suggested (2) that different parts of the Fc region (3–5) of the immunoglobulin may provide points of attachment for the Clq subcomponent of complements (17). Thus, a possible explanation for the reduction in anticomplementary activity of human IgG1 upon modification of the arginine residues is that such residues are necessary for the binding of the complement to the immunoglobulin.

The synthesis of peptides and polypeptides that mimic the sequences shown in Table II and their anticomplementary activities will be reported later.

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