Dissociation of Aggregated IgG and Denaturation of Monomeric IgG by Acid Treatment

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UEMURA, Y. *Dissociation of Aggregated IgG and Denaturation of Monomeric IgG by Acid Treatment.* Tohoku J. exp. Med., 1983, **141** (3), 337-349 — Conditions for obtaining a large quantity of monomeric IgG which can be used for intravenous injections in humans was successfully established with human serum IgG preparation obtained using the ethanol fractionation method. When dissociation of IgG aggregates and denaturation of IgG monomers contained in the starting material were examined at various pH values, the treatment for 60 min at 28°C at pH 3.8-4.0 was found to be optimum for obtaining such monomeric IgG. At this pH range, the dissociation of IgG aggregates into monomeric IgG reached maximum, without producing polymeric IgG. When monomeric IgG was treated at this acidic pH condition, its biological and physicochemical properties such as acid difference spectrum, molecular weight, plasmic digestion ratio, and complement-, antigen- and protein A-binding activities remained unchanged. The fact that large scale production of monomeric IgG can be easily performed under such conditions is discussed. ——— dissociation; denaturation; human IgG; acid treatment

Conventional intramuscular immunoglobulin (IgG) preparations obtained by cold ethanol or ammonium sulfate fractionation methods contain IgG aggregates. The aggregates have some biological activities such as anticomplemental and antigenic activities (Ishizaka and Ishizaka 1959; Christian 1960; Henney and Ellis 1968) and may evoke anaphylactoid reactions, particularly in immunodeficient patients. Therefore, its intravenous use has to be strictly avoided.

Several methods have been reported for the elimination or dissociation of the aggregates to obtain IgG preparations suitable for intravenous use. Acid treatments is one of the methods for the dissociation of the aggregates. Earlier experimental results (Hansson 1968) showed that aggregated human IgG was dissociated by acid treatment at pH 3.0 in the presence of glycine. But the question was, to what extent did such acid treated IgG retain its biological as well as physicochemical properties. Analysis of human IgG by circular dichroism (CD) (Doi and Jirgensons 1970) showed that IgG after acid treatment at pH 2.2 differed slightly from untreated IgG. Further studies on the biological properties of IgG under other pH conditions have not been performed. Biological properties

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of acid treated rabbit IgG were studied at various pH conditions by Stollar et al. (1976). In their report, complement binding activity decreased after treatment at pH 3.0 but the antigen binding activity survived after the same treatment.

Since rabbit IgG differs from human IgG in its sensitivity to proteases (Hsiao and Putnam 1961) and in the properties of its fragments (Fey 1975), the author attempted to study the biological and physicochemical properties of human monomeric and aggregated IgG molecules before and after acid treatment in detail. The purpose of this paper is to describe the molecular weight, acid difference spectrum, sensitivity against plasmin, and complement-, antigen- and protein A-binding activities of human IgG before and after acid treatment at various pH values.

MATERIALS AND METHODS

Human IgG

Human IgG was isolated from normal human plasma by the cold ethanol fractionation method.

HBsAg

Purified HBsAg was obtained using both polyethylene glycol (PEG) fractionation and ultracentrifugation (Funakoshi et al. 1981).

Lipopolysaccharide

Lipopolysaccharide (LPS) of *Pseudomonas aeruginosa* F-1 was purified by the phenol-water extraction method (Westphal et al. 1952).

Complement

Lyophilized guinea pig serum complement was purchased from Kyokuto Seiyaku, Tokyo.

Human plasmin

Plasminogen purified from Cohn's Fr-III paste of human plasma by the lysine-Sepharose method (Deutsch and Mertz 1970) was activated by passage through the urokinase-conjugated Sepharose column. The proteolytic activity of the plasmin was determined by the caseinolytic method (Sgouris et al. 1961)

Gel filtration

Isolation of IgG aggregates and IgG monomer fractions was performed on a Sephadex G-200 column (2.5 × 90 cm). Six ml of 5% IgG in 0.075 M phosphate buffer (pH 7.0) containing 0.075 M NaCl (PBS) was passed through the column at a flow rate of 25 ml/hr and at 4°C. Fraction was collected in 6.7 ml and the absorbance at the 280 nm was determined with a Shimadzu UV-240 spectrophotometer. Analysis of the relative amount of IgG aggregates and the monomer was done by the high performance liquid chromatography (HPLC) method. SWP (7.5 cm) was used as a precolumn and 3,000 SW (120 cm) was used as a fractionation column (Toyo Soda Manufacturing, Tokyo). Both SWP and 3,000 SW were equilibrated with 0.05 M acetate buffer containing 0.3 M NaCl, pH 6.5. A sample solution (25 μ l) was passed through the columns at a flow rate of 1 ml/min.

Acid treatment

To 3 ml of IgG solution (1.4–7.0 mg/ml) in 0.05 M KCl containing 0.2% PEG #4,000, 0.1 ml of HCl solution (up to 0.1 N) was added for pH adjustment. After the acid treatment,

the IgG solution was neutralized with NaOH solution or neutral buffer solution.

Difference spectrum

Difference spectra were recorded with a Shimadzu automatic recording spectrophotometer model UV-240. The pH of IgG monomer solution (1.4 mg/ml) in test tubes was adjusted serially from 1.6 to 7.0, and all the test tubes were allowed to stand for 60 min at 25°C. The absorbance of the pH-treated solution at a given pH was read against the same solution at pH 1.6. Then the pH of the solution was recorded with a Hitachi-Horiba pH meter model F-7.

Plasmin digestion

To 1 ml of IgG solution in PBS (2.8 mg/ml), 0.1 ml of plasmin solution (5 casein units/ml) or the same volume of PBS (for blank test) was added and the mixture was incubated at 28°C for 3.5 hr. After incubation, an equal volume of ammonium sulfate solution (80% saturated, pH 7.0) was added and the mixture allowed to stand at 4°C for 30 min followed by centrifugation at 3,000 rpm for 10 min to obtain a clear supernatant. Absorbances at 280 nm (A₂₈₀ nm) of the supernatant and the original IgG solution were measured and the amount digested was calculated by the following formula:

$$\text{Plasmin digestion (\%)} = \frac{\text{A}_{280 \text{ nm}} (\text{after digestion}) - \text{A}_{280 \text{ nm}} (\text{before digestion})}{\text{A}_{280 \text{ nm}} (\text{original IgG})} \times 100$$

Antigen-antibody complex

Acid treated IgG (pH 7.3, 0.36 mg/ml) was mixed with an equal volume of the antigen solution at an optimum concentration and incubated at 37°C for 1 hr and then at 4°C for another hr. Immune complex thus prepared was tested for anticomplementary activity.

Anticomplementary activity

Sucrose gelatine veronal buffer (SGVB), pH 7.3, was prepared as follows: 5.5-diethylbarbituric acid sodium salt (1.019 g), NaCl (4.98 g), sucrose (39.126 g), gelatine (1.0 g), CaCl₂·2H₂O (22.0 mg), MgCl₂·6H₂O (101.5 mg) and N-HCl (3.5 ml) in a volume made to total 1 liter with distilled water.

A sample solution (0.4 ml), 2 units guinea pig complement (0.4 ml) and SGVB (1.8 ml) was mixed in a test tube and the mixture was incubated for 60 min at 37°C. Then, sensitized erythrocytes in SGVB (0.4 ml) were added to the test tube and the tube was incubated with gentle stirring for 60 min at 37°C. After incubation, cells were pelleted and the supernatant was monitored spectrophotometrically (541 nm) for hemoglobin release. Anticomplementary activity was calculated by the following formula and expressed in % of hemolysis inhibition:

$$\% \text{ of hemolysis inhibition} = \frac{\text{A}_{541 \text{ nm}} (\text{IgG}) - \text{A}_{541 \text{ nm}} (\text{IgG} + \text{antigen})}{\text{A}_{541 \text{ nm}} (\text{buffer})} \times 100$$

Antigen binding activity

Antigen binding activity was assayed by the parallel line passive hemagglutination (PHA) method using antigen sensitized sheep red blood cells (Uemura et al. 1980).

Chromatography on Staphylococcus protein A

Protein A Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) was packed in a column and washed with PBS, pH 7.0. On a column containing 0.7 ml of gel, 7 mg of IgG was loaded at a flow rate of 3 ml/hr, and unabsorbed IgG was recovered by washing with PBS.

Protein concentration

The absorbance at 280 nm in 1 cm light pass was measured. The absorbance of IgG solution was 1.40 per mg protein determined by Kjeldhal's method and this factor was applied to all samples in estimating protein concentration.

RESULTS

Separation of aggregated and monomeric IgG fractions

Aggregated and monomeric IgG fractions were obtained by gel filtration. Fig. 1 shows the results of a typical experiment. As designated in Fig. 1, aggregated fractions and monomeric fractions were pooled individually. Analysis of the pooled fractions by HPLC showed that the monomeric fraction consisted of

Fig. 1. Gel filtration of human IgG on Sephadex G-200. Human IgG preparation for intramuscular use was applied on a column (2.5×90 cm) at a flow rate of 25 ml/hr and at 4°C . Fractions of 6.7 ml were collected and the absorbance at 280 nm was determined. Aggregate and monomer fractions indicated by arrows were pooled individually.

Fig. 2. Immunoelectrophoresis of the aggregated and monomer fractions isolated by gel filtration using anti-human serum (rabbit).

98% monomer and 2% dimer, and the aggregated fraction consisted of 38–55% monomer and 62–45% dimer. In the immunoelectrophoretic test using anti-human serum (rabbit), the monomeric fraction revealed a single and IgG specific precipitin line and no detectable traces of any other proteins (Fig. 2). The aggregated fraction revealed two precipitin lines, i.e. one was identical to IgG and the other to IgA. The concentration of IgA contaminated in the aggregated IgG fraction was 3% of the total protein. The aggregated and monomeric fractions thus obtained were dialyzed against 0.05 M KCl containing 0.2% PEG, pH 7.0, for 24 hr at 4°C and diluted with the same solution to obtain a suitable IgG concentration for the following studies.

Dissociation of IgG aggregates by acid treatment

The pH of the IgG aggregate fraction (5.7 mg/ml) was adjusted to 2.7–6.9 with HCl, incubated for 60 min at 28°C and then neutralized with NaOH. The acid treated IgG solutions were diluted with equal volumes of PBS, pH 7.0, and tested for HPLC and plasmin digestion. The aggregated fraction used in this study consisted of 38% monomer, 61% dimer and 1% polymer before acid treatment. As shown in Fig. 3, lowering pH resulted in an increase in monomer content and a decrease in dimer content. However, the polymer content increased apparently at pHs below 3.8. Plasmin digestion was performed after the neutralization. Fig. 3 shows that the percentage digestion was minimum at a pH range of 3.8–4.0 and higher at pH values below 3.8 or above 4.0, where the concentrations of polymer or dimer were higher than those at pH 3.8–4.0. In the

Fig. 3. Dissociation of IgG aggregates by acid treatment. Aggregate fractions were treated at various acidic pHs at 28°C for 60 min and neutralized.

Monomer (—○—), dimer (—●—) and polymer (---○---) concentrations, and plasmin digestion ratio (---◇---) were determined.

Fig. 4. Time-course of dissociation of IgG aggregates at pH 3.8. IgG aggregate fractions were adjusted to pH 3.8 at 28°C, and diluted five times with PBS for neutralization at indicated times. Concentration of IgG monomer (□) and aggregates (▨) were determined by the HPLC method.

next experiment, time course of the dissociation at acidic pH was studied. The pH of the aggregated fraction in the test tube was adjusted to 3.8 at 28°C and a 0.2 ml portion was transferred into 5 times this amount of PBS for neutralization. The percentage contents of monomer and aggregates as revealed by the HPLC method are shown in Fig. 4. Before acid treatment, the contents of monomer and aggregates in the aggregated fraction were 55% and 45%, respectively. The monomer content increased to 77% after 1 min and reached a plateau within 30 min, but about 13% of the aggregates still remained after 360 min. Judging from the results so far obtained, it may be concluded that the optimum pH range for the dissociation of IgG aggregates is 3.8–4.0, and that the dissociation occurs rapidly and reaches a plateau after 30 min. To determine whether the acid treated IgG undergoes denaturation or not, the acid stability of human IgG was studied.

Acid difference spectrum

Fig. 5 shows typical difference spectra of human IgG monomer fraction at indicated pHs against the same solution at pH 1.6. Two peaks and one shoulder was observed at 292 nm, 285 nm and 278 nm, respectively. The height of the two peaks at 292 nm and 285 nm increased with increasing pH. Fig. 6 shows the values of absorbance of the peak at 292 nm as a function of pH. The values fell sharply at pH below 3.8 indicating acid denaturation. The reversibility of the acid denaturation of IgG monomer was studied as follows: The IgG monomer fraction (1.4 mg/ml) was adjusted to pH 2.4–5.2 and allowed to stand for 60 min at 25°C, and then neutralized with NaOH. After additional incubation for 20 hr at 4°C, the absorbance was measured against a protein solution at pH 1.6, at 25°C. The absorbance values at 292 nm were similar to those obtained with protein that was not exposed to acid prior to the measurement.

Fig. 5. Acid difference spectra of IgG monomer.

Reference: IgG monomer in 0.05 M KCl containing 0.2% PEG (pH 1.6). 1, pH 5.4; 2, pH 3.2; 3, pH 2.7.

Fig. 6. Absorbance at 292 nm as a function of pH. The values measured at a given pH and at neutral pH after exposure to a given pH are shown by closed and open circles, respectively. Reference: IgG monomer in 0.05 M KCl containing 0.2% PEG (pH 1.6).

Change in molecular weight

An IgG monomer fraction (1.4 mg/ml) was incubated at acidic pHs for 60 min at 28°C, neutralized with NaOH and allowed to stand for 20 hr at 4°C. Turbidity was observed macroscopically at pH below 3.0. The molecular weight and concentration of IgG monomer remained unchanged at pH 3.3–7.0, but IgG monomer concentration decreased to 70% and a significant amount of polymer appeared at pH below 3.0 (Fig. 7).

Plasmin digestion

An IgG monomer fraction (5.7 mg/ml) was incubated at acidic pH and neutralized as described above. Plasmin digestion obviously accelerated at pHs

Fig. 7

Fig. 7. Change in molecular weight after acid treatment. IgG monomer fractions were treated at various pHs for 60 min at 28°C and neutralized. The monomer (—○—), dimer (—●—) and polymer (—·—) concentrations were measured by the HPLC method.

Fig. 8

Fig. 8. Plasmin digestion ratio after acid treatment. IgG monomer fractions were treated at various pHs for 60 min at 28°C and neutralized. One (—●—) and 24 (---○---) hr after neutralization, plasmin digestion tests were performed.

Fig. 9. Change in complement binding activity after acid treatment. Acid treated IgG solution (pH 7.3) was incubated with the corresponding antigen solution at an optimum concentration to form immune complex. The anticomplementary activity of the complex was then measured.

below 3.5, and the plasmin digestion-pH pretreatment curve obtained 24 hr after neutralization shifted slightly to acidic range as compared to that 1 hr after neutralization (Fig. 8). That human IgG monomer undergoes denaturation below pH 3.5–3.7 was also confirmed by subsequent studies.

Complement binding activity

IgG monomer fractions (3.6 mg/ml) were obtained from hyperimmune anti-HBs and anti-LPS globulin preparations. The two IgG monomer fractions were treated individually at acidic pHs for 60 min at 28°C and diluted 10 times with SGVB for neutralization. The acid-treated hyperimmune IgG solutions (0.36 mg/ml) were incubated with the corresponding antigen solutions at optimum concentrations to produce the immune complexes. Fig. 9 shows that anticomplementary activities of the immune complexes were constant during the test over the pH range of 3.7–7.0, but sharply decreased at pH below 3.6 indicating the denaturation of IgG monomer at the Fc region.

Antigen binding activity

Acid treatment of IgG monomers was performed by a method similar to that reported in Fig. 9. As judged by their ability to bind antigen which were

Fig. 10. Change in antigen binding activity after acid treatment. IgG monomer fraction was treated at various pHs for 60 min at 28°C and neutralized. Remaining antigen binding activity was measured by the parallel line PHA method.

sensitized on red cells, acid treatment at pH 3.7 did not affect the antigen binding sites, viz., Fab regions. In case of anti-HBs globulin, the activity did not decrease even after pH 3.0 treatment (Fig. 10).

Protein A binding activity

Since the absorbing capacity of the protein A-Sepharose column used in my experiment was found to be about 10–15 mg/ml gel, 7 mg of acid treated IgG was applied on the column, and the amount of unabsorbed IgG that might correspond to the sum of IgG₃ and denatured IgG was determined by the single radial immune diffusion method. As shown in Fig. 11, the binding ability of IgG to protein A was not affected by acid treatment at pH above 3.7.

Fig. 11. Change in protein A binding activity after acid treatment. Acid treated IgG solution (pH 7.0) was applied on a protein A-Sepharose column (7 mg IgG/0.7 ml gel) and washed with PBS. IgG concentration in the unadsorbed fraction was measured by the single radial immune diffusion method.

DISCUSSION

Intramuscular IgG preparation used in the present study contained about 30% aggregated IgG and this percentage conformed to a previous report (Römer et al. 1982). The aggregates in the IgG preparation was thought to be formed by denaturation occurring at ethanol fractionation and freezing-thawing in the purification processes.

Though ideal dissociation of the aggregated IgG was found to occur at pH 3.8–4.0, a part of the aggregates still remained after the acid treatment. The author suppose that the persistent aggregates are composed mainly of deep-denatured IgG, but further studies are required to explain this phenomenon.

In acid difference spectrum studies, Bence Jones protein showed three peaks at 279, 286 and 293.5 nm (Takahashi et al. 1970). But, in the case of human IgG monomer, the absorbance at 279 nm was not clear and seen only as a shoulder. The change in the absorbance at 292 nm with pH illustrated in Fig. 6 showed that human monomer IgG underwent acid denaturation at pH below 3.8, but the denaturation determined by this method was reversible.

The molecular weight of human IgG measured at pH 3.0 was smaller than 7S, but it returned to 7S after neutralization and a small amount of insoluble materials appeared in the IgG solution (Hansson 1968). The human IgG monomer fraction

showed turbidness after acid treatment at pH below 3.0, too, and the supernatant contained polymers indicating denaturation. However, the molecular weight of the IgG treated at pH 3.3–7.0 was identical to that of the native one. The relationship between IgG subclasses and the tendency to denature or polymerize during acid treatment will be examined in the future.

In general, the sensitivity of proteins to protease increases after denaturation (Linderström-Lang et al. 1938). For example, rabbit IgG in native form is resistant to plasmin but the sensitivity increases after acid denaturation (Connel and Porter 1971; Stewart and Stanworth 1975). Since the sensitivity of human IgG monomer to plasmin increased after acid treatment below pH 3.7 as illustrated in Fig. 8, the IgG might undergo some denaturation by acid treatment, and the denaturation might be slightly reversible at neutral pH.

Besides antigen binding activity, complement binding activity is one of the important biological properties of immune globulins. Fab and F(ab')₂ have antigen binding but not complement binding activities, so the bacteriolytic activity in the presence of complement are remarkably inferior to that of native IgG (Doi et al. 1978). Studies on acid treatment of rabbit IgG showed that the antigen binding activity survived but not the complement binding activity (Stollar et al. 1976; Winkelhake et al. 1980). In the present study, the antigen binding activity of human IgG monomers survived acid treatment at pH 3.0–3.5 but the complement binding activity was reduced by the treatment. This phenomenon may correlate with the phenomenon that Fab and F(ab')₂ fragments were more stable than Fc fragment in acidic condition (Abaturov et al. 1969; Charlwood and Utsumi 1969).

When anti-HBs and anti-LPS globulins used in the present studies were separated individually into electrophoretically slow and fast IgG fractions by an ion exchange chromatography method (Uemura et al. 1975), the anti-HBs and anti-LPS activities were distributed mainly in the slow and fast IgG fractions, respectively (data not shown). This suggests that the slow IgG (anti-HBs) and fast IgG (anti-LPS) may have similar stability in acidic conditions.

IgG₁, IgG₂ and IgG₄ but not IgG₃ bind to protein A at C_H3 and C_H2 domains (Stewart et al. 1978). When the Fc region of IgG was modified chemically with β -propiolactone, the binding activity decreased appreciably (Skvaril et al. 1980; Ceska 1981). Decrease in binding activity of acid treated IgG at pH below 3.7 to protein A also means acid denaturation. In other words, acid treatment at pH above 3.7 does not denature human IgG.

The biological half-life of acid treated IgG (pH 4) was similar to that of IgG, but those of sulfonated and β -propiolactone treated ones were shorter than that of the above two (Morell et al. 1980). These in vivo results also suggest that acid treatment at pH 3.8–4.0 does not alter the properties of human IgG.

On the basis of these results, the author concludes that a pH value of 3.8–4.0 is reasonable for the dissociation of aggregated IgG and that human IgG does not undergo denaturation at this condition.

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