Studies on the Interaction between Protein A and Immunoglobulin G: I. Effect of Protein A on the Functional Activity of IgG

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STUDIES ON THE INTERACTION BETWEEN PROTEIN A AND IMMUNOGLOBULIN G

I. Effect of Protein A on the Functional Activity of IgG

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Staphylococcal protein A (PA) and IgG anti-Forssman immunoglobulin formed complexes that behaved functionally like IgM in their ability to lyse sheep erythrocytes (E) in the presence of whole guinea pig complement (GPC) and to fix purified guinea pig C1. Concanavalin A, a plant lectin that inhibited IgM but not IgG hemolytic activity, inhibited the hemolytic activity of IgG-protein A complexes that behaved like IgM but had no effect on complexes that behaved functionally like IgG. Since Con A is known to bind specifically to glucose and mannose residues, our results suggested that the interaction of protein A with the Fc region of IgG led to exposure of sugar moieties that may participate in complement (C) binding. The production of IgM-like complexes depended on the ratio of protein A to IgG and the empirical formula of these IgM-like complexes was found to be [(IgG)2PA]n. As the ratio of PA to IgG was increased, the resulting complexes tended to behave functionally like IgG but with reduced hemolytic activity and C1 fixing ability. Furthermore, the binding of C1 to IgG was inhibited by PA and the binding of PA to IgG was inhibited by C1 indicating that the binding sites for C1 and PA were located near each other or were identical. Our results offer a reasonable explanation for the reported effects of PA or mixtures of PA and IgG in vitro and in vivo.

Protein A (PA), isolated from the cell wall of Staphylococcus aureus has been found to bind specifically to the Fc fragment of IgG isolated from several species including human IgG subclasses 1, 2, and 4 (1, 2). PA interacting with IgG produced immunologic effects including complement (C) fixation (3–6) in vitro and a hypersensitivity reaction when injected into experimental animals and man (7, 8). Much of the work aimed at elucidating the mechanism responsible for these immunologic phenomena has centered on the effect that mixtures of PA and IgG have on C activity. It has been shown that whole serum C can be activated or inhibited depending on the dose of PA, and that complexes between PA and either IgG or the isolated Fc fragments of IgG depleted human serum of late (e.g., C8, C9) as well as early acting C components (5, 6).

In this report we present the results of experiments designed to study the direct effect of PA on the functional activity of IgG hemolytic antibody to Forssman antigen. The effect of PA on Forssman antibody depended on the ratio of IgG to PA. At the lowest ratios of IgG to PA, inhibition of hemolytic activity was observed. This effect was attributable to the competitive inhibition of binding of C1 to IgG by PA. At higher ratios, PA interacted with IgG to produce complexes that behaved functionally like IgM antibody in their ability to fix C.

Concanavalin A (Con A), a lectin isolated from the Jack bean, was found to inhibit the functional activity of IgG-PA complexes that behaved like IgM but had no significant effect on the activity of a mixture of IgG and PA that behaved functionally like IgG alone. We have reported that Con A inhibited the hemolytic activity of IgM but not IgG (9). The effect of temperature or varying the cell number on the hemolytic activity of the IgM-like complexes also was determined.

Based on the concentration of IgG in the antibody preparations (determined by a protein binding assay (10)), and hemolytic data, we calculated that two IgG molecules were required per PA molecule to give a complex that behaved like IgM. The ability of this complex, represented by the empirical formula [(IgG)2PA]n, to fix C more efficiently than IgG may offer a reasonable explanation for the diverse immunologic effects observed for mixtures of PA and IgG.

MATERIALS AND METHODS

Staphylococcal PA was purchased from Pharmacia Fine Chemicals, Piscataway, N. J. Con A was obtained from Sigma Chemical Co., St. Louis, Mo. The preparation of 125I-PA and the development of the protein binding assay used to quantify IgG immunoglobulin have been reported (10).

Buffers. Isotonic Veronal-buffered saline, pH 7.4, containing 0.1% gelatin, 0.001 M Mg++, and 0.00015 M Ca++ (VBS-gel) was prepared according to the method of Rapp and Borsos (11).

Cells. E were collected and washed as described by Kabat and Mayer (12).

Antibody. Antisera to Forssman antigen were raised in New Zealand albino rabbits according to the procedures given in (12). The IgG fractions were isolated by chromatography on DEAE cellulose (13). The fraction rich in IgM was prepared by chromatography of the antisera on Sephadex G-200 (14).

C and C components. Guinea pig complement (GPC), purchased from JEM Research, Inc., Kensington, Md., and human
C that was absorbed with E (12) were stored in 1-ml aliquots at -30°C. Functionally pure GPC1 was purchased from Cordis Corp., Miami, Fla.

Determination of C1. Bound C1 was determined on a molecular basis by the C1 fixation and transfer test (C1FT) described in (11).

Hemolytic assay. E (generally 0.1 ml of 1.5 × 10⁸ cells/ml) that were sensitized by incubation with an appropriate dilution of antibody at 30°C for 30 min were treated with excess GPC (i.e., the degree of hemolysis depended only on the concentration of antibody) for 60 min at 37°C in a total volume of 1.5 ml Veronal-buffered saline (VBS)-gel. The tubes were centrifuged and the degree of hemolysis was determined by comparing the absorbance of the supernatant fluids at 412 nm to that of a sample of cells lysed completely by addition of water.

RESULTS

Effect of PA on the hemolytic activity of IgG anti-Forssman antibody. The IgG fraction of rabbit antiserum (No. WG377) to Forssman antigen was isolated by chromatography on a column of DEAE-cellulose (13). To test the effect of PA on hemolytic antibody activity, serial dilutions of antibody (0.05 ml) were incubated for 30 min at 30°C with an equal volume of either VBS-gel or a solution containing either 0.0033 or 0.0049 mg PA/ml buffer. These dilutions were used to sensitize an equal volume (0.1 ml) of E (1.5 × 10⁸ cells/ml) by a second similar incubation. Each cell suspension was then treated with excess GPC at 37°C for 60 min before the degree of hemolysis was determined. The results in Figure 1A show that in the presence of PA, the titration curves are biphasic. At relatively high ratios of IgG to PA, the slope is approximately 1.0, which is characteristic of IgM (15, 16). At lower ratios the slope changes to 4.4, the same as the slope obtained for the antibody in the absence of PA (Fig. 1), and significant inhibition of hemolytic activity was observed. To determine if PA had the same effect on other antisera, we tested the IgG fractions from two other antisera from rabbits immunized with sheep cell stroma. The results are presented in Figure 1B for the IgG fraction from one rabbit (No. WG371). In addition to the biphasic response observed with No. WG377 (Fig. 1A), an additional change in the slope was observed. At the highest IgG to PA ratios, the mixtures behaved like IgG. As the ratio decreased, the mixtures behaved like IgM and then again like IgG. Similar triphasic patterns were observed with the third IgG sample (HC32, data not shown). Since the lysis of E/IgG by C in the presence of PA (data not shown) was not affected, PA does not modify C activity under these conditions.

Our next experiments were designed to examine the two segments of the titration curve for serum No. 377 (solid circles; Fig. 1A); the first in which the mixture of IgG and PA behaved essentially like the control sample of IgG, except the titer was lower, and the second in which the Ig-GA mixture behaved like IgM.

Effect of PA on lysis of E at high or low ratios of PA to IgG. A ratio of PA to IgG that corresponded to a point on the IgG-like part of the titration curve (solid circles) shown in Figure 1A was prepared. This mixture was less active hemolytically than comparable amounts of antibody in buffer alone. E (1.5 × 10⁸) sensitized with dilutions of this mixture (i.e., a constant ratio of PA to IgG) or with antibody diluted with VBS-gel were tested for their ability to fix GPC1 by the C1FT described in (11). As shown in Figure 2, this dose of PA inhibited C1 fixation to IgG. This inhibition correlated to the inhibition of hemolytic activity observed under the same conditions (Fig. 1). The slope of the C1 fixation curve was the same (2.2) for the control sample of antibody prepared in buffer and for this mixture of PA and IgG. In another experiment, E (0.1 ml of 10⁶ cells/ml)
were sensitized with sufficient IgG that had produced complete hemolysis if excess GPC were added. E prepared in this manner were washed free of unbound antibody, and aliquots of the cell suspensions were incubated for 15 min at 37°C with differing amounts of PA. The cells were washed and then treated with excess GPC. The number of C1 molecules fixed in each cell preparation was determined by the CIPT (11). The curves in Figure 3A show that inhibition of C1 fixation to E1gG was dependent on the dose of PA. At the highest level of PA tested, C1 fixation was inhibited by 42%. Thus, binding of C1 was inhibited either by treating IgG with PA in the fluid phase before sensitization of E or after the IgG is cell-bound.

We also used 125I-labeled PA (125I-PA) to show that C1 bound to E1gG inhibits the binding of PA. In this experiment, 1.5 × 10^7 E (0.1 ml) were sensitized with differing amounts of IgG anti-Forssman antibody, washed with VBS-gel, then treated with buffer or with excess GPC. The cells were washed and the binding of 125I-PA (10,700 cpm added) to aliquots of each cell suspension (5 × 10^6 cells) was determined under conditions that we have shown to be optimal (10). The curves in Figure 3B show the binding of 125I-PA to E1gG over the range of antibody concentrations tested and that C1 bound to E1gG effectively inhibited the binding. However, inhibition was observed only at those relatively high concentrations of antibody that caused hemolysis in the absence of PA. Similar results were obtained when human complement (HuC1) was used (data not shown). As reported previously (10), 125I-PA did not bind to E1gM or E1gMC1.

A high ratio of IgG to PA that corresponded to a point on the IgM-like part of the titration curve (solid circles) shown in Figure 1A was prepared. Dilutions of this mixture (i.e., constant ratio of PA to IgG) were used to sensitize 1.5 × 10^7 E (0.1 ml). The cells were washed and then incubated with sufficient C1 to saturate the C1 binding sites. The number of C1 molecules fixed to the cells was determined by the CIPT (11). The slope of the curve shown in Figure 2 obtained by using dilutions of this IgM-like mixture was 0.8, close to the value of 1.0, characteristic of IgM. The slope of the standard IgG curve was 2.2.

![Figure 3](http://www.jimmunol.org/)

Figure 3. A, inhibition of C1 binding to E1gG by differing amounts of PA. E1gG were prepared from sufficient anti-Forssman IgG that would give complete lysis in the presence of GPC. The cells were incubated with VBS-gel or with VBS-gel containing differing amounts of PA, and the number of C1 molecules bound to each cell preparation determined by the CIPT (11). A relative concentration of t = 0.038 mg PA/ml, B, inhibition of binding of PA to E1gG by C1. E (1.5 × 10^7) were sensitized with differing amounts of IgG anti-Forssman antibody, washed, then treated with either VBS-gel, or with excess GPC1 to give E1gG or E1gGC1, respectively. A relative IgG concentration of 1 = 1/16. The ability of E1gG (O—O) or E1gGC1 (Δ—Δ) to bind 125I-PA (10,700 cpm added) was determined as described in the text. The hemolytic dose-response curve obtained for E1gG in the presence of whole GPC also is shown (■—■).

Effect of cell number, Con A, or temperature on hemolytic activity of PA-IgG mixtures.

Next we determined how mixtures of IgG and PA behaved under conditions where IgG and IgM would be expected to behave differently, i.e., the effect of temperature, Con A, or variation in cell number on the antibody activity was tested. In one experiment, a mixture of PA and Ig anti-Forssman antibody that fell on the IgM-like part of the titration curve shown in Figure 1A was prepared. Dilutions of this mixture (i.e., a constant PA to IgG ratio) were used to sensitize equal volumes (0.1 ml) of cell suspensions containing either 3.0, 2.0, 1.5, or 1 × 10^7 E/ml. After incubation with excess GPC for 1 hr at 37°C, the degree of hemolysis was measured. The slopes of the dose-response curves shown in Figure 4A were approximately 1.0. The ratio of the antibody dilutions required to give an average of one lytic site per cell was 4.0:2.6:1.7:1.0 compared to the cell number ratio of 3.0:2.0:1.5:1.

Figure 4. A, effect of variation in cell number on the titration of an IgM-like mixture of IgG anti-Forssman antibody and PA. Serial dilutions of an IgM-like mixture of IgG and PA (0.004 mg/ml) were used to sensitize 3.0 (O—O), 2.0 (■—■), 1.5 (Δ—Δ), or 1.0 × 10^7 E (□—□). Hemolysis was determined as described in the text. A relative antibody concentration of 1 = 1/10. B, effect of concanavalin A on the hemolytic activity of IgG-anti-Forssman antibody and mixtures of IgG and PA. Serial dilutions of an equimolar mixture of IgG and PA were used to sensitize E to lysis by GPC as described in the text. A relative IgG concentration of 1 = 1/6.4.

The IgG-PA complexes behaved ideally as IgM antibody, the ratio of the titers and the ratio of the number of cells used in the titrations would be the same. This deviation from ideal behavior may indicate the presence of free IgG and/or other hemolytically active species.

Con A inhibited the hemolytic activity of IgM anti-Forssman antibody whereas IgG activity was either unaffected or enhanced by Con A (9). To determine the effect of Con A on the hemolytic activity of the IgM-like complexes, dilutions of an appropriate mixture of PA and IgG were incubated for 30 min at 30°C in the presence of 0.06 mg Con A/ml or in the presence of VBS-gel. The results shown in Figure 4B show that Con A inhibited the hemolytic activity of the IgM-like IgG-PA com-
plexes but enhanced slightly the hemolytic efficiency of the control sample of IgG. This effect apparently does not result from an interaction between Con A and PA since the IgG-like titration curve obtained in the presence of a relatively large dose of PA was not affected by the lectin (Fig. 4B).

We also tested the effect of temperature on the hemolytic activity of PA-IgG mixtures. It is known that IgG fixes C1 more effectively at 4°C than at 37°C, whereas IgM binds C1 more efficiently at 37°C than at low temperature (17). Consequently, the hemolytic activity of IgG is increased by incubation with C at 4°C followed by incubation at 37°C. Dilutions of antibody (0.05 ml) were mixed with equal volumes of either 0.022 or 0.067 mg PA/ml buffer or with VBS-gel, and incubated at 30°C for 30 min. These solutions were used to sensitize 10^8 E (0.1 ml) at 37°C for 30 min, then cold VBS-gel (0.5 ml) was added. Aliquots (0.1 ml) of each cell suspension were incubated with excess GPC in a total volume of 1.5 ml buffer either at 4°C or 37°C for 30 min. Both sets of tubes were then incubated at 37°C for 1 hr and the degree of hemolysis determined. (The same results were obtained if this incubation was carried out for 90 min.) The titration curves are shown in Figure 5. The hemolytic activity of the IgG diluted in VBS-gel and treated with GPC in the cold was significantly higher than the activity of the antibody treated with C at 37°C. The activity of the antibody diluted in the presence of either concentration of PA also was greater when preincubated with GPC at 0°C, although the increase was not as marked. The activity of the IgG-PA mixtures that fell on the IgM-like part of the titration curve (Fig. 5) also was increased.

**Empirical formula of the IgM-like complex.** The concentration of 7S immunoglobulin in each of the IgG fractions isolated from three antisera was measured by the protein binding assay described in (10). By the use of these values, the m.w. of PA (42,000) and of IgG (160,000), and by knowing the concentration of PA used, we could calculate the ratio of IgG molecules per PA molecule at any point in titration curves like those shown in Figure 1. The results in Table I indicate that the lowest ratio of IgG:PA that behaved like IgM was close to 2:1 for the three IgG preparations. Thus the empirical formula [(IgG)_2PA]_n gives the minimum number of IgG molecules per PA that will give a complex that behaves like IgM.

**Figure 5.** Effect of temperature on the hemolytic activity of IgG anti-Forssman antibody and mixtures of IgG and PA. To dilutions of IgG was added an equal volume of either VBS-gel (C, O), 0.022 mg PA/ml (▲, △), or 0.067 mg PA/ml (◆, ▽). Aliquots of each set of dilutions were used to sensitize E. These cells were incubated with GPC at either 4°C (C, △, ◆) or 37°C (O, ▲, ▽) for 30 min. After an additional 1 hr incubation at 37°C, the degree of hemolysis was determined. Other details are given in the text. A relative IgG concentration of 1 = 1/6.4.

### TABLE I

<table>
<thead>
<tr>
<th>IgG Preparation</th>
<th>Concentration of IgG (mg/ml)</th>
<th>(IgG/PA)</th>
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</thead>
<tbody>
<tr>
<td>WG371</td>
<td>0.12</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>WG377</td>
<td>0.48</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>HC32</td>
<td>0.10</td>
<td>1.7 ± 0.1</td>
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* Rabbit IgG fraction isolated from rabbit antiserum to Forssman antigen by chromatography on DEAE cellulose (see Materials and Methods).

* Concentration of IgG determined by the protein binding assay reported in Reference 10.

* The minimum number of IgG molecules per PA molecule necessary to form an IgM-like complex. The values represent the mean ± S.E. based on data (see Fig. 1 for representative curves) obtained for two different concentrations of PA. The molecular weights of IgG and PA were taken to be 160,000 and 42,000 daltons, respectively.

### DISCUSSION

The specific binding of staphylococcal PA to the Fc region of IgG has been the basis of several methods of isolating (18) and quantifying IgG (10, 19, 20). In addition, mixtures of PA and IgG have been shown to fix C in vitro (3-6) and to produce immediate hypersensitivity reactions in vivo (7, 8). These effects depend on the ratio of PA to IgG.

In this paper we have reported results of experiments designed to measure the direct effect of PA on the hemolytic activity of rabbit anti-Forssman IgG under conditions where C activity was unaffected by PA. The hemolytic dose-response curve of the IgG fraction isolated by chromatography on DEAE cellulose was linear with a slope of 4.4 (Fig. 1), a value consistent with IgG (15, 16). In contrast, the dose-response curve in the presence of a constant amount of PA was bi- or triphasic. At lower antibody concentrations the slope was the same as that of the control (4.4) but at higher concentrations approached 1.0, a value characteristic of IgM (15, 16). We examined these two regions of the dose-response curve in detail for one IgG preparation. The lowest IgG:PA ratios behaved functionally as IgG. However, the titer was reduced in a way that correlated with an inhibition of C1 fixation to E that were sensitized with the PA-IgG mixture. Similarly, C1 fixed to E1gG inhibited the binding of ^125I-PA.

Inhibition of PA binding to E1gG by cell-bound C1 was observed only at the concentrations of antibody that gave extensive hemolysis. This result was consistent with the proposal (21) that at least one site consisting of two or more molecules of IgG on the cell surface was required to fix C1. Humphrey (22) estimated that approximately 800 molecules of IgG must be bound to an erythrocyte to produce one doublet. Below this threshold, C1 will have no influence on the ability of PA to bind to E1gG. Also, our results are consistent with the
suggestion of Kronvall and Gewurz (5) that the binding sites for PA are close to those for Cl on the Fc region of IgG. Steric hindrance of Cl binding by PA bound to IgM may account for the inhibition of C fixation at high ratios of PA to IgG. No effect of PA on the fixation of Cl to IgM antibody was observed (data not included). Similar to PA, the supernatant fluids of inhibited C1 binding by PA bound to IgG may account for the inhibition of C fixation at high ratios of PA to IgG. No effect of PA on the fixation of Cl to IgM antibody was observed (data not included). Similar to PA, the supernatant fluids of the IgM-like part of the titration curve.

We also found that Con A inhibited the hemolytic activity of the IgM-like complex, but not a mixture of PA and IgG that behaved like a control sample of IgG. We showed earlier (9) that the lectin inhibits IgM but not IgG anti-Forssman antibody. Since Con A binds specifically to glucose and mannose residues (24), these sugar moieties may become exposed on the IgG molecule once it is bound to PA and play a role in the functional activity (i.e., C fixation) of the IgM-like complex as well as of IgM. In this regard, it has been shown that significant conformational changes occur in the IgG molecule when it is bound to PA (25). Since IgG is known to contain glucose and/or mannose (26), and PA contains no significant carbohydrate (27), the sugar(s) must be part of the IgG. The effects of specific sugar-degrading enzymes on the activity of the IgG-like complex, compared to IgM and IgG, may give further insight into the functional role of these sugar moieties.

The correlation between the titers of an IgM-like mixture of IgG and PA, and the number of E used in the titrations was close to the 1:1 ratio expected for IgM (11). The observed discrepancy may reflect the presence of more than one hemolytically active species, e.g., free IgG. For IgG antibody, a 1:1 relationship between cell number and antibody titer was not observed (Borsos, unpublished results).

We have analyzed the composition of the IgG-PA complexes that behaved like IgM. Based on the concentration of IgG in the antibody preparations and by using the m.w. of PA (42,000), we determined the ratio of IgG to PA at points on titration curves shown in Figure 1. Our calculations indicated that the IgM-like complex contained two IgG molecules per PA molecule. The same results were obtained for IgG preparations from different rabbit sera (Table I). Thus the empirical formula [(IgG)2PA], gives the minimum number of IgG molecules per PA in a complex that behaves like IgM. Molecule weight determinations based on the sedimentation profiles of these complexes on sucrose gradients suggested that they have a molecular formula of (IgG)2PA.2. These experiments will be reported in detail (28), along with evidence that not all the IgG molecules in the complex must be directed against the Forssman antigen.

These results may offer a reasonable explanation for several of the immunologic effects reported to be associated with PA. The depletion of C activity from whole serum (3, 5, 6) and the Arthus-like reaction induced in humans or experimental animals by injection of PA (7, 8) can be interpreted in terms of efficient C fixation by IgM-like complexes formed between IgG and PA. Less C fixation observed at relatively high doses of PA (3, 5) is expected since these conditions favored the predominance of complexes in which the IgG-PA ratio approached one, and such complexes may be less efficient in fixing C than IgG alone. Consistent with this hypothesis, the slope of the titration curve in Figure 1 was characteristic of IgG in the presence of high doses of PA and the titer of the antibody was reduced.

REFERENCES


