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Research Article

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Release of Serotonin from Human Platelets Induced by Aggregated Immunoglobulins of Different Classes and Subclasses

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ABSTRACT The ability of human myeloma proteins of different classes and subclasses and of macroglobulins (all aggregated with bis-diazotized benzidine or heat) to aggregate washed human platelets and release [³H]-serotonin from the platelets was investigated and compared with the activity of normal IgG and tetanus-anti-tetanus IgG antigen-antibody complexes. Aggregated IgG1, IgG2, IgG3, IgG4, and normal IgG complexes all aggregated platelets and caused release of serotonin to similar extents. In contrast, IgA1, IgA2, IgD, and IgE myeloma proteins as well as IgM macroglobulins were completely inactive in this respect. Approximately 50% of the activity remained in aggregated, mildly reduced and alkylated IgG myeloma proteins and their Fc fragments, whereas aggregated F(ab')₂ fragments were completely inactive. Addition of fresh serum inhibited the release of serotonin caused by aggregated IgG1 and IgG3 proteins and normal IgG antigen-antibody complexes by about 50% but had no effect upon the release of serotonin obtained with IgG2 and IgG4 proteins. This inhibition appeared to be mediated by complement. The release of serotonin was not accompanied by liberation of the cytoplasmic enzyme lactic dehydrogenase, indicating that no significant lysis of the platelets occurred. Addition of neutrophils did not enhance the serotonin release.

INTRODUCTION

Platelets from all mammalian species studied interact with antigen-antibody or antigen-antibody-complement

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complexes, causing the platelets to adhere to the complexes and stimulating platelet aggregation (1-7). The aggregation of the platelets has been shown to result in part from release of ADP from the adhering platelets (8). The adherence and aggregation of the platelets also lead to release of a portion of their vasoactive substances, such as serotonin (4-11). The mechanism of this reaction appears to differ in different species. In rabbits, the platelets adhere to immune complexes and aggregate only when the third component of complement (C3) is bound to the complexes (1-3, 10), whereas in man complement does not appear to be required for this reaction (3-7). The release of serotonin from human platelets induced by reaction with immune complexes may result from an active secretory process and is not a result of lysis (5, 7, 12, 13).

The classes and subclasses of immunoglobulins responsible for this stimulation of human platelets are unknown. The experiments reported here were performed to investigate this question and to define further the mechanism by which aggregated immunoglobulins act on human platelets.

METHODS

Platelets. Venous blood from normal human volunteers was taken into 1/7 the volume of acid citrate dextrose (ACD)¹ (14). The platelets were prepared according to the method of Mustard, Perry, Ardlie, and Packham (15). Platelet-rich plasma was removed after centrifugation (220 g, 20 min) and was incubated for 15 min at 37°C with 0.2 μCi/ml [³H]serotonin (Amersham/Searle Corp., Arlington Heights, Ill.). The platelets were sedimented (1000 g, 15 min) and washed first with solution 1 (see below) then with solution 2. They were then resuspended in solution 3,

¹ *Abbreviations used in this paper:* ACD, acid citrate dextrose; BDB, bis-diazotized benzidine; BSA, bovine serum albumin; LDH, lactic dehydrogenase; PBS, phosphate-buffered saline.

in which the reactions were performed. All preparations were carried out at room temperature. Solution 1 consisted of Tyrode's solution of pH 6.35 containing 2 mM MgCl₂ but no Ca⁺⁺, 3.5 g/liter bovine serum albumin (BSA) (Pentex, Miles Laboratories, Inc., Kankakee, Ill.), 20,000 U/liter hirudin (Sigma Chemical Co., St. Louis, Mo.), and 100 mg/liter apyrase (Sigma Chemical Co.). Solution 2 was identical with solution 1 but contained no hirudin. Solution 3 consisted of Tyrode's solution containing 1.4 mM CaCl₂, 3.5 g/liter BSA, and 100 mg/liter apyrase, pH 7.35. In some experiments, [ethylenebis(oxyethylenenitrilo)]tetracetic acid (EGTA, 0.2 mM) was employed in solution 1. Aggregate-free platelet suspensions were standardized to 3 × 10⁸/ml turbidometrically and in most experiments checked for their ability to aggregate upon addition of ADP in the presence of human fibrinogen (Nutritional Biochemicals Corp., Cleveland, Ohio). Neutrophils were prepared by techniques described previously (16).

Antiserum. Human antitetanus antiserum was obtained from an individual 14 days after a tetanus toxoid "booster" injection. The globulin fraction was precipitated with 50% saturated ammonium sulfate and the IgG, used as the source of antibody, isolated by DEAE-cellulose column chromatography using 0.01 M phosphate buffer, pH 8.0. Tetanus-antitetanus toxoid precipitates were prepared at equivalence and washed before use.

Isolation of proteins. A total of 31 myeloma proteins, 3 macroglobulins, and normal IgG were studied. The myeloma proteins consisted of 4 IgG1, 4 IgG2, 4 IgG3, 8 IgG4, 4 IgA1, 2 IgA2, 3 IgD, and 2 IgE immunoglobulins. IgG myeloma proteins of slow electrophoretic mobility were isolated from the sera of patients with multiple myeloma by DEAE-cellulose chromatography using 0.01 M phosphate buffer, pH 8.0. Normal IgG was similarly obtained from Cohn fraction II. IgG myeloma proteins of fast γ or β electrophoretic mobility and IgA myeloma proteins were isolated by Pevikon block electrophoresis (17) followed by Sephadex G-200 gel filtration. IgD myeloma proteins were isolated as previously described (18). IgE myeloma proteins were purified by DEAE-cellulose chromatography with 0.015 M phosphate buffer, pH 8.0, followed by Sephadex G-200 gel filtration. IgM macroglobulins were isolated by repeated euglobulin precipitation followed by Sephadex G-200 gel filtration. The isolated proteins were analyzed by immunoelectrophoresis using sheep anti-whole human serum (Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.). Only traces of contaminating β and α_2 proteins were detected in the IgA and IgM preparations, respectively. The IgG preparations showed a single precipitating line suggesting that no complement components were absorbed on them. In order to minimize the amount of normal IgG in the IgG myeloma proteins, whenever possible sera from patients having 4 g or more myeloma protein per 100 ml of serum were used. The class and the light chain type were determined by double diffusion in agar by using specific rabbit or goat antisera prepared according to the method of Fahey and McLaughlin (19). The IgG subclasses were identified by using specific rabbit antisera (20). When the IgG myeloma proteins were analyzed at 1 mg/ml by double gel diffusion with specific antsubclass antisera, they reacted with only one antiserum, indicating that contamination with other subclasses was less than 5%, since 50 μ g IgG/ml was detected with these antisera. F(ab')₂ and Fc fragments of IgG myeloma proteins were prepared as previously described (21) with the exception of IgG2. Those Fc fragments were isolated after digestion with papain in the presence of 0.1 M mercaptoethanol for 1 h at 37°C. IgG and

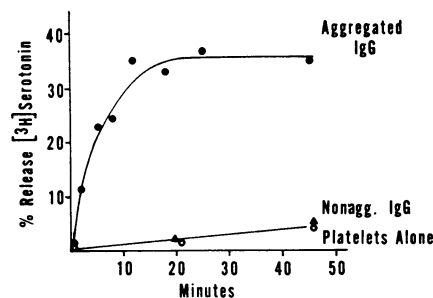


FIGURE 1 The release of serotonin from washed human platelets stimulated by BDB aggregates of normal IgG (insoluble aggregates). Platelets (3×10^8) were incubated with 250 μ g aggregates or nonaggregated IgG for varying times at 37°C. The reaction was stopped by cooling to 4°C and the percent release of [³H]serotonin determined.

IgA myeloma proteins were partially reduced in 0.5 M Tris buffer, pH 8.2, at a concentration of 0.02 M dithiothreitol for 1 h at room temperature and alkylated by addition of 0.05 M recrystallized iodoacetamide for 30 min in the cold. The reduced and alkylated proteins were then dialyzed against phosphate (0.01 M)-buffered 0.15 M NaCl, pH 7.0 (PBS).

Aggregation of proteins. The myeloma proteins, macroglobulins, normal IgG, and IgG fragments were aggregated either with bis-diazotized benzidine (BDB) or by heating. Aggregation with BDB was performed according to the method described by Ishizaka, Ishizaka, Salmon, and Fudenberg (22). Routinely, 5 mg of protein dissolved in 2 ml of 0.15 M borate buffer, pH 8.5, were aggregated at 0°C by the addition of freshly prepared BDB, diluted 1:10 with borate buffer, and the pH was maintained at 8.5 by addition of 0.1 N NaOH. After incubation for 30 min in the cold, the preparations were dialyzed against PBS. The degree to which individual myeloma proteins and their fragments aggregated with a given amount of BDB varied greatly. The amount of BDB necessary to aggregate the myeloma proteins to form either soluble or insoluble aggregates was, therefore, determined in preliminary tests by addition of 50–150 μ g of BDB. Addition of large quantities of BDB resulted in the formation of predominantly insoluble aggre-

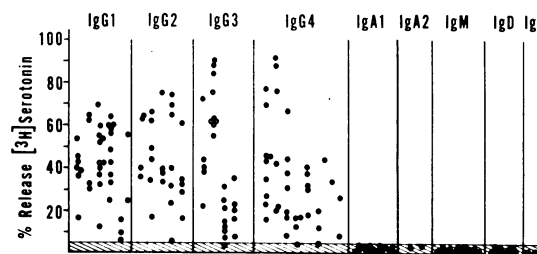


FIGURE 2 Release of serotonin from human platelets incubated with BDB-aggregated myeloma proteins of different classes and subclasses. Each column represents one myeloma protein, and each point, the mean of duplicate determinations employing platelets of different donors. The platelets were incubated with 250 μ g of either insoluble aggregates (solid circles) or soluble aggregates (open circles) for 30 min at 37°C, and the percentage of released [³H]serotonin was determined. The shaded area indicates one standard deviation from the mean release from platelets incubated alone.

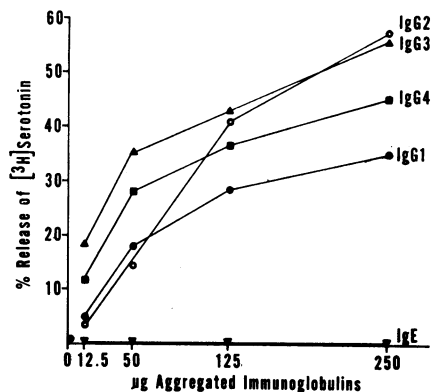


FIGURE 3 Release of serotonin from platelets stimulated with increasing quantities of insoluble aggregates of individual myeloma proteins.

gates, which were disbursed to form a turbid solution before use. Addition of smaller amounts of BDB yielded a clear, yellow solution which consisted of relatively small aggregates sedimenting faster than the untreated proteins and appeared as a broad peak when analyzed by sucrose gradient density ultracentrifugation. These aggregates will be called soluble aggregates.

The myeloma proteins were also aggregated by heating at a concentration of 5 mg protein/ml PBS in a 63°C water bath for 5–30 min. The time of incubation was varied, depending upon the speed of visible aggregation.

Experimental conditions and assays. Platelets (0.1 ml, i.e., 3×10^8) were incubated for 30 min at 37°C with the aggregated immunoglobulins (usually 250 µg) or immune complexes suspended in 1 ml of solution 3. After mixing and incubation with mild shaking, the platelets were cooled, centrifuged for 15 min at 1800 g, and the fluid was assayed for [³H]serotonin in a Beckman liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). The percent release was calculated from the total [³H]serotonin in the platelets obtained by lysis of the platelets with Triton X-100 (1 mg/ml). All points were determined in duplicate. When serum was included in the reaction, 10% fresh normal human serum containing 10 U/ml hirudin was added to both the experimental and control tubes.

β-Glucuronidase (E.C.3.2.1.31) and lactic dehydrogenase (LDH) (E.C.1.1.1.27) were assayed and the release expressed as a percentage of that extractable from the platelets with Triton X-100 as described previously (10). The total amounts of these enzymes did not change during the incubation. Aggregation of platelets suspended in solution 3 containing 1 mg/ml fibrinogen was measured at 37°C while the mixture was stirred at 1,100 rpm with an aggregometer (Chrono-Log Corp., Broomall, Pa.). The release of adenine nucleotides was determined by measuring the absorption of 260 nm of HClO₄ extracts which had been neutralized with KOH (6).

RESULTS

Release of serotonin induced by aggregated normal human IgG. The release of serotonin from washed human platelets incubated for increasing lengths of time with 250 µg of insoluble BDB-aggregated normal human IgG is shown in Fig. 1. The maximum release was

reached after 25 min incubation with aggregated IgG. Nonaggregated IgG did not liberate serotonin. An incubation time of 30 min was therefore chosen for all subsequent experiments.

Release of serotonin induced by aggregated myeloma proteins of different classes and subclasses. The ability of aggregated myeloma proteins of different classes and subclasses and of macroglobulins aggregated with BDB to release serotonin from platelets is shown in Fig. 2. As can be seen, aggregated IgG1, IgG2, IgG3, and IgG4 myeloma proteins were able to stimulate release of the amine. In contrast, aggregated IgA1, IgA2, IgD, and IgE myeloma proteins and IgM macroglobulins were completely inactive. Both soluble and insoluble BDB-aggregated proteins (see Methods) were tested. In general, insoluble aggregates of IgG myeloma proteins were more effective in releasing serotonin than soluble aggregates. Unaggregated IgG proteins were all inactive. The amounts of serotonin released using the same preparation of aggregated myeloma proteins varied for different platelet donors.

The percent release of serotonin induced by increasing amounts of aggregates is shown in Fig. 3. While a similar threshold was observed for each individual protein, each exhibited different activities. For example, one of the IgG3 protein (second column under IgG3 in Fig. 2) consistently released more serotonin than other IgG3 proteins; whatever the degree of aggregation or the source of the platelets, such a variation was seen within all four IgG subclasses.

In order to demonstrate that BDB aggregation was not unique in conferring the ability or inability of immunoglobulins to release serotonin from platelets, heat-aggregated proteins were also tested. The results depicted in Fig. 4 show that the release of serotonin from platelets by myeloma proteins of the different classes and subclasses aggregated by heat was similar to that obtained with BDB-aggregated proteins. The heated IgG4 proteins were somewhat less active than the IgG1, 2, and

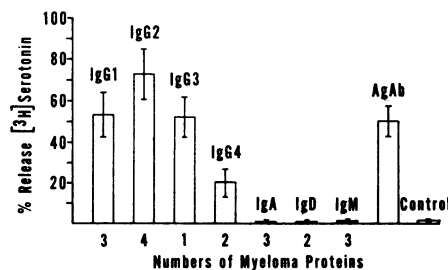


FIGURE 4 Release of serotonin from platelets incubated with heat-aggregated myeloma proteins and antigen-antibody complexes. The mean percent release \pm SEM induced by 250 µg aggregated proteins and 50 µg tetanus-antitetanus antigen-antibody precipitates (AgAb) is shown. The control represents platelets incubated alone.

3 proteins; however, this was probably not significant since the proteins available for study aggregated poorly when heated. The effect of 50 μ g tetanus-antitoxin precipitates also shown in Fig. 4 demonstrates that about one-fifth the amount of IgG antigen-antibody complexes was necessary to obtain a release similar to that by 250 μ g of heat-aggregated IgG myeloma proteins.

Aggregation of platelets. Representative samples of experiments demonstrating the aggregation of platelets after addition of BDB-aggregated IgG myeloma proteins and of an IgM macroglobulin (250 μ g) are shown in Fig. 5. Similar aggregation was observed with all four subclasses of IgG but with no other class of immunoglobulins. Release of ADP (the percentages being similar to those of released serotonin (e.g. 58% serotonin with 50% ADP) was detected after addition of the aggregated IgG myeloma proteins to the platelets; however, the aggregation induced by the proteins did not appear to be due entirely to ADP release, since it was not inhibited by addition of adenosine.

Release of serotonin induced by aggregated, reduced, and alkylated IgG myeloma proteins and by fragments of these proteins. The effect on platelets by aggregated, reduced IgG and fragments of IgG proteins was investigated in order to obtain information on the submolecular site(s) responsible for stimulating release of serotonin. As shown in Fig. 6, mild reduction and alkylation before aggregation resulted in a 25–75% loss of activity of the IgG1, 2, 3, and 4a proteins tested and a complete loss of a single IgG4b protein available for study. Fc fragments retained an activity similar to that found for the reduced and alkylated proteins; Fc fragments of the IgG4b protein were also inactive. All F(ab)₂ fragments examined were completely negative.

The effect of serum on the release of serotonin and the release of platelet enzymes. Experiments were performed to determine whether the presence of 10% serum as a

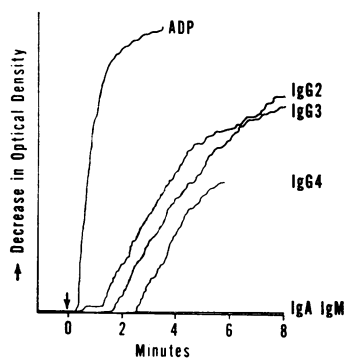


FIGURE 5 Aggregation of platelets after addition of BDB-aggregated myeloma proteins and of an aggregated IgM macroglobulin as compared with the effect of 5×10^{-4} M ADP. Decrease in optical density measured in the aggregometer indicates aggregation of platelets.

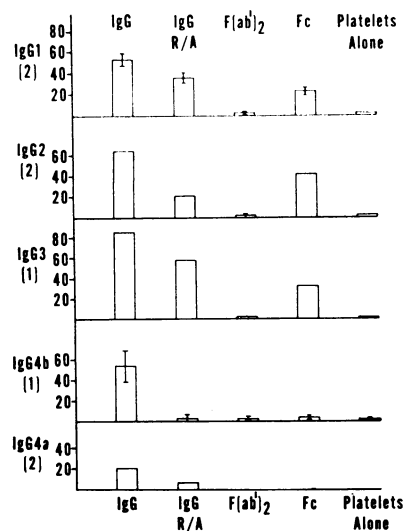


FIGURE 6 Release of serotonin from platelets incubated with 250 μ g of soluble aggregates of IgG, mildly reduced and alkylated (R/A) IgG, and fragments of IgG myeloma proteins. The number of different proteins from which fragments were tested is shown in parentheses. (From top down) (a) Intact IgG myeloma proteins, (b) reduced and alkylated (R/A) IgG, (c) F(ab)₂ fragments, (d) Fc fragments, and (e) platelets alone. All proteins of which the SEM is given were tested with platelets of three to four different donors and the others with platelets from one donor.

source of complement has an effect on the release of serotonin induced by aggregated immunoglobulins. It has been shown that lysis of rabbit platelets can be induced by reaction with rabbit immune complexes and complement (10, 13). Therefore, release of the cytoplasmic enzyme LDH and of the granule enzyme β -glucuronidase was determined. The experiments depicted in Fig. 7 show that addition of fresh normal serum inhibited the release of serotonin by IgG1 and IgG3 by more than 50% but had no effect on the release resulting from addition of IgG2 and IgG4 proteins. Heat inactivation of the serum (56°C, 30 min) abolished the inhibitory activity on IgG1 and IgG3 proteins. Heat-inactivated serum did not induce release by itself. Tetanus-antitoxin antibody complexes (25 μ g) stimulated 22% serotonin release in this particular experiment. In the presence of fresh serum, the release was reduced to 12.5% but was unchanged when heat-inactivated serum was used. The presence of fresh serum did not confer releasing activity upon the IgA or IgM aggregates (not shown in Fig. 7). As shown in Fig. 7, addition of aggregated IgG1 and IgG2 myeloma proteins did not result in significant release of LDH or β -glucuronidase, indicating that they did not cause lysis of the platelets.

The effects of neutrophils on the release of serotonin. Rabbit neutrophils previously have been shown to aug-

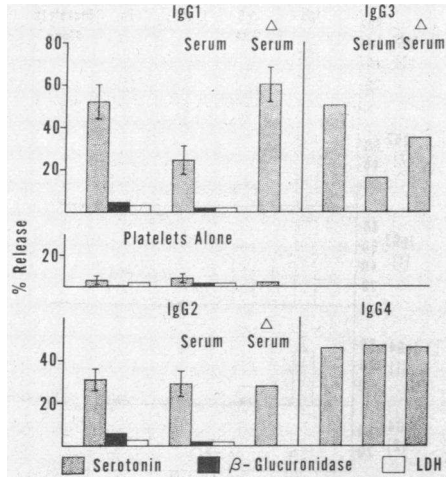


FIGURE 7 The effect of fresh and heated (Δ) serum on the release of serotonin, β -glucuronidase, and LDH from human platelets. Aggregated myeloma proteins ($250 \mu\text{g}$) were incubated with washed platelets in the presence of 10% human serum. 1 U of hirudin was included in all tubes to inhibit any thrombin in the serum. In the control tubes (the first set of bars in each group), although serum was omitted, hirudin was nevertheless included. The mean percentage of release of serotonin \pm SEM and of β -glucuronidase and LDH for four experiments is shown.

ment the immune complex-induced release of vasoactive amines from rabbit platelets (11) by mechanisms which are as yet unclear. However, no augmenting effect on the release of serotonin from human platelets was observed when 1×10^4 – 1×10^6 neutrophils were added to 3×10^8 platelets stimulated with $50 \mu\text{g}$ tetanus-antitetanus complexes (to yield 57.9% release).

DISCUSSION

The results of these studies demonstrated that aggregated human immunoglobulins of all four IgG subclasses caused aggregation of platelets and release of serotonin from the platelets. In contrast, aggregated IgA, IgD, IgE, and IgM immunoglobulins were completely inactive in this respect. In the absence of serum, no significant difference was found between IgG proteins of different subclasses. In contrast, addition of fresh serum as a source of complement resulted in about 50% inhibition of serotonin release with IgG1 and IgG3 proteins but not with IgG2 and IgG4 proteins. These findings suggest that in man only IgG antigen-antibody complexes activate platelet functions *in vivo* and that IgG2 and IgG4 may play a more important role than their concentration in serum would suggest.

The action on platelets was shown by a number of control experiments to result from the aggregated Ig proteins and not from contaminating substances. Nonaggre-

gated IgG myeloma proteins did not activate platelets, indicating that any traces of thrombin or endotoxin which might have been present in these preparations were not responsible for the activity. Moreover, hirudin, an inhibitor of thrombin, did not prevent the release of serotonin.

To exclude the possibility that BDB aggregation was conferring some property on IgG proteins but not on other immunoglobulins, heat-aggregated proteins were also studied. Again, only aggregated IgG proteins were active. That similarly prepared IgA aggregates were biologically active, although not on platelets, was shown by their ability to release granule enzymes from neutrophils (23) and to activate the alternate pathway of C fixation (24). As shown by the dose-response curves, the effect on platelets could not be the results of contamination of one subclass by another. The threshold dose for activation of platelets was the same, about $12.5 \mu\text{g}$, for all four subclasses. Furthermore, the content of normal IgG in the myeloma proteins was estimated by double gel diffusion tests to be less than 5% and could therefore not account for all the activity. Individual myeloma proteins differed in their activity within all four subclasses. These differences might be the result of variations in the extent to which the proteins could be aggregated to form biologically active complexes or they might reflect a structural heterogeneity. Similar heterogeneity within a given subclass has also been observed in studies of the catabolism of IgG myeloma proteins (25) and of fixation of complement (22).

The portion of the IgG molecule responsible for stimulation of the platelets appeared to be the Fc fragment. Aggregated Fc fragments from all but one IgG myeloma protein tested induced release of serotonin, but aggregated $F(ab')_2$ fragments did not. While it is possible that $F(ab')_2$ fragments could no longer form biologically active aggregates in contrast to Fc fragments, it was nevertheless clear that the latter fragments could by themselves induce the release. Mild reduction and alkylation of the IgG proteins before aggregation reduced their ability to stimulate release of serotonin. This suggests that configurational integrity is required for optimal reaction with platelets. It is possible that reduced and alkylated proteins aggregate differently from unaltered molecules. Since the proteins are being reduced during digestion with papain, it was not surprising that the activity recovered in the Fc fragments did not exceed that of the reduced and alkylated IgG proteins. Interestingly, one IgG4b protein was completely inactive after reduction and alkylation and its Fc fragment was also inactive. It is presently not known if the loss of activity is related to the 4a and 4b genetic marker (26), since no additional 4b proteins were available to test this possibility.

Addition of fresh serum to the medium partially inhibited the release of serotonin caused by normal IgG, IgG1, and IgG3 proteins but not by IgG2 and IgG4 proteins. Similar inhibition has previously been observed by Mueller-Eckhardt and Lüscher (7) for normal IgG, which is known to consist mainly of IgG1. This inhibition is probably the result of complement components which bind to the IgG aggregates. The inhibition paralleled the ability of the subclasses to fix complement by the classical pathway (22), and the inhibitory factor(s) was (were) heat labile. Further studies involving purified complement components are necessary to clarify this point. Alternatively, unaggregated immunoglobulins in the serum might compete for receptors on platelets and prevent them from adhering to the aggregated proteins. This appears very unlikely since heating of the serum at 56°C for 30 min would not be expected to prevent this inhibition and, moreover, attempts to block the reaction of aggregated myeloma proteins with nonaggregated proteins have so far been unsuccessful.

Human and rabbit platelets differ in their reaction with immunoglobulin complexes. It has been shown that rabbit platelets adhere to aggregated immunoglobulins only after fixation of the complement component C3 to the aggregate (1-3) and that addition of neutrophils enhances the release of vasoactive amines (11). As shown in this study, complement was not necessary to induce release of serotonin from human platelets although at this time the possibility that the immunoglobulin is reacting with C1 absorbed on the platelet cannot be excluded. This point is under investigation. The addition of neutrophils did not enhance the release, contrasting with the effect of these cells in the rabbit system (11). The above-mentioned reaction of rabbit platelets in the presence of the whole complement sequence (at least through C6) results in complement-dependent lysis of the platelets. No such lysis was observed with the human platelet. In contrast, the release of serotonin described here appears to be an active secretory process, probably similar to the so-called release reaction of platelets (13). These differences may be explained by the suggestion that there are receptors for C3 on rabbit platelets which are absent in human platelets, while in contrast human platelets appear to have receptors for the Fc portion of IgG.

Addendum. After completion of this manuscript, a study by S. L. Pfueller and E. F. Lüscher (1972. The effects of aggregated immunoglobulins on human blood platelets in relation to their complement-fixing abilities. I. Studies of immunoglobulins of different types. *J. Immunol.* **109**: 517.) was reported which describes results on the release of serotonin from platelets similar to ours. These authors also showed that myeloma proteins of all four IgG subclasses react with platelets to cause release of serotonin, whereas IgA proteins did not; IgM, IgD, and IgE proteins were not examined. It was shown in this study that the comple-

ment component C1q inhibits release of serotonin caused by normal IgG, which is in agreement with our finding that serum used as a source of complement inhibits the release of IgG1 and IgG3 which together make up about 75% of normal IgG. Pfueller and Lüscher found a low activity in three IgG3 proteins and a great variation in the activity of five IgG4 proteins. In the present study a variation in activity was found within all subclasses (Fig. 2) and therefore no significant differences between the four different subclasses could be demonstrated. The variation within a subclass is presently unexplained. It appears most likely that the variation in activity is the result of differences of different proteins to form biologically active complexes after aggregation by BDB or heat and does not reflect differences between the subclasses.

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