Pneumococcus-Induced Serotonin Release from Human Platelets

IDENTIFICATION OF THE PARTICIPATING PLASMA/SERUM FACTOR AS IMMUNOGLOBULIN

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ABSTRACT Pneumococcus-induced serotonin release from human platelets is greatly facilitated by a factor present in normal human plasma and serum. We have identified this factor as immunoglobulin by: (*a*) removing it from plasma and serum with solid phase anti-Fab antibody; (*b*) demonstrating its absence from the serum of an individual with severe immunoglobulin deficiency; and, (*c*) showing its presence in IgG preparations isolated from normal individuals. Evidence suggesting that the release reaction is triggered by pneumococcal antigen-antibody complexes rather than by nonimmune interaction of immunoglobulin with pneumococcus includes: (*a*) the failure of isolated IgG myeloma proteins to support release; (*b*) a lack of correlation between IgG concentration and "releasing factor activity" in normal human sera; (*c*) the identification of a normal serum that supports release by types II and III pneumococci but not by type VII; and, (*d*) the ability of most normal sera to support release by pneumococcal serotypes II and VII, though these types have not shown nonimmune reactivity with the Fc portion of the IgG molecule. The ability of antibodies present in normal serum to support pneumococcus-induced serotonin release suggests that the thrombocytopenia seen in pneumococcal infection may at least in part be caused by pneumococcal antigen-antibody complexes.

INTRODUCTION

Thrombocytopenia, sometimes accompanied by thrombohemorrhagic complications, frequently accompanies infection with different strains of microorganisms (1–3). Though the mechanism of this thrombocytopenia is often not understood, it has been shown that several strains of bacteria can induce human platelets to aggregate and/or cause release of serotonin and nucleotides with the participation of a plasma/serum factor or factors (4–6). We have identified the factor that interacts with pneumococcus to induce release of serotonin from human platelets as immunoglobulin. Our studies indicate that the release is induced by pneumococcal antigen-antibody complexes and that antibodies capable of supporting this reaction are present in the great majority, if not all, of normal human plasma and sera. This reaction between pneumococcus, antibody, and platelets may play an important etiologic role in the thrombocytopenia and/or thrombo-hemorrhagic (7–9) complications accompanying infections with this microorganism.

METHODS

Preparation of [^{14}C]serotonin labeled platelets. The method of preparing washed platelets was modified from methods described previously (10, 11). Venous blood (45 ml) was taken into 5 ml of acid citrate dextrose (ACD formula A) in 50-ml conical plastic culture tubes. The cellular constituents of the blood were separated from plasma by centrifugation at 2,300 *g* for 15 min at ambient temperature (22–25°C) and then suspended in a volume of modified Tyrode's buffer equal in volume to the plasma volume. 40 μCi of [^{14}C]serotonin (New England Nuclear, Boston, Mass.) were added to the 50-ml cell suspension and the mixture was incubated at 37°C for 30 min. The cellular constituents of the suspension were then removed by centrifugation at 2,300 *g* for 15 min and washed four times by resuspension in Tyrode's buffer. The cell mixture was then resuspended in sufficient buffer to reconstitute the mixture to the initial whole blood volume. The platelets were then separated from the other cellular elements by slow centrifugation at 190 *g* for 15 min, and the small number of red and white cells that remained were removed by centrifugation at 620 *g* for 60 s.

Tyrode's buffer was modified in the following ways: the initial suspending buffer and that used for washing contained 0.03 M adenosine to prevent aggregation. Adenosine was omitted from the final suspending buffer. Calcium was omitted from all but the final suspending buffer. It was also omitted from the final suspending buffer if plasma (as

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opposed to serum or isolated protein preparations) was used in the succeeding experiments. Bovine serum albumin was used at a concentration of 5% in the initial suspending buffer and at a concentration of 2% thereafter. Platelet counts of the final suspension varied between 200,000 and 600,000/mm^3 with less than 0.1% of contaminating red or white cells present.

Preparation of platelet-rich plasma, plasma, and sera. Normal platelet-rich plasma, plasma, and sera were obtained from volunteer donors between the ages of 21 and 40 yr who gave no history of previous pneumococcal infections or of any respiratory infections within the previous 2 mo.

Platelet-rich plasma was prepared from citrated whole blood by centrifugation at 190 *g* for 15 min, and contained 3–5 $\times$ 10^5 platelets/mm^3. Plasma was prepared from citrated whole blood by centrifugation at 2,300 *g* for 15 min. Serum was prepared by allowing whole blood to clot 2 h at 37°C. The clot and formed elements were removed by centrifugation at 2,300 *g* for 15 min. Plasma and sera were used fresh or stored at −70°C.

IgG concentrations of normal sera were determined by radial immunodiffusion. The immunoglobulin G, A, D, and M concentrations of the serum of an individual with severe pan immunoglobulin deficiency were determined to be below 10 μg/ml by double diffusion. None of these proteins were detected with monospecific antisera capable of detecting purified preparations of these proteins at concentrations of 10 μg/ml.

Isolation of immunoglobulins. Normal IgG was prepared by precipitating the globulin fraction of a pool of three normal sera or of the serum of a single normal donor with ammonium sulfate and isolating the IgG by DEAE cellulose chromatography with 0.01 M phosphate buffer at pH 8.0. Alternatively, normal IgG was isolated from Cohn fraction II (American Red Cross) by the same method. IgG myeloma proteins of slow electrophoretic mobility were isolated directly from serum by DEAE cellulose chromatography with 0.005 M phosphate buffer at pH 8. IgG myeloma proteins of fast gamma or beta electrophoretic mobility were isolated by Pevikon block electrophoresis (Mercer Consolidated Corp., Yonkers, N. Y.) followed by Sephadex G-200 gel filtration (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). When the isolated proteins were analyzed by immunoelectrophoresis at a concentration of 20 mg/ml with sheep anti-whole human serum (Hyland Division, Travenol Laboratories, Inc., Costa Mesa, Calif.), no serum proteins other than IgG were detected. To minimize the amount of normal IgG in the IgG myeloma proteins, whenever possible sera from patients having 4 g or more myeloma proteins/100 ml of serum were used. The class and light chain type were determined by double diffusion in agar by using specific rabbit antisera prepared according to the method of Fahey and McLaughlin (12). The IgG subclasses were identified with specific rabbit antisera (13).

F(ab)$_2$ and Fc fragments of normal IgG were prepared as previously described (14). All immunoglobulins had been dialyzed against 0.15 M sodium chloride and were diluted at least one part to four parts in Tyrode's buffer before use.

Fibrinogen was provided by Drs. Thomas Edgington and Edward Plow. It was prepared as previously described (15), was 95% clottable, and gave one band on sodium dodecyl sulfate polyacrylamide electrophoresis. It was dialyzed against phosphate-buffered saline before use (0.01 M Na_2PO_4, 0.15 M NaCl, pH 7.3).

Preparation of pneumococci. Three different isolates of pneumococci, type III were used; these were provided by Dr. Edward Goldzimmer, Oceanside, Calif.; by Dr. Robert Austrian, Department of Research Medicine, University of Pennsylvania, School of Medicine, Philadelphia, Pa.; and by Mr. John Cortney, Department of Microbiology, Washington University, School of Medicine, St. Louis, Mo. Dr. Robert Austrian also provided isolates of types II and VII. Pneumococci were subcultured on blood agar plates in a 5% CO_2 atmosphere. They were passed through mice if their activity in these experiments appeared to be decreasing and/or if they began to lose their capsules. For use in these studies, the pneumococci were grown for 16–18 h at 37°C in Trypticase soy broth in a 5% CO_2 atmosphere and harvested by centrifugation at 27,000 *g* for 30 min, rewashed four times in barbital-buffered saline, and suspended in approximately 1/20 of the culture volume of normal saline. Pneumococci were then heated for 1 h at 100°C to inactivate pneumolysin and stored at −70°C. Before use they were diluted to the appropriate concentration in barbital-buffered saline (usually 8 $\times$ 10^8/ml).

Preparation of solid phase anti-F(ab)$_2$ antibody (anti-(Fab')$_2$ beads). Anti-F(ab')$_2$ sera were prepared in rabbits as described previously (16) and the globulin fraction isolated by precipitation with 50% saturated ammonium sulfate. The antibody globulin was coupled to Sepharose 2B beads (Pharmacia Fine Chemicals) by cyanogen bromide as described elsewhere (16*a*). The globulin fraction from a pool of normal rabbit serum was similarly coupled to Sepharose 2B beads for use as a control.

Depletion of "releasing factor" from diluted plasma and serum was accomplished by incubating equal volumes of anti-F(ab)$_2$ beads or control beads and diluted plasma or serum for 4 h at room temperature with constant rocking. The beads were then removed by centrifugation at 7,000 *g* for 5 min.

Release of radiolabeled serotonin from human platelets. Release of [^{14}C]serotonin from radiolabeled platelets was determined in the following manner. 25 μl of platelet suspension was incubated with 200 μl of either plasma, serum, isolated protein preparations, or Tyrode's buffer or dilutions of plasma, sera, or isolated proteins in the modified Tyrode's buffer used for the final platelet suspension. 20 μl of bacterial suspension or barbital-buffered saline was then added. Incubations were routinely continued for 15 min without stirring. Platelets were then removed by centrifugation at 7,000 *g* for 5 min and 200 μl of the supernate were then placed in 10 ml of Aquasol (American Cyanamid Co., Wayne, N. J.) and counts per minute were determined in the Packard Tricarb liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). The 100% platelet [^{14}C]serotonin was determined by counting 200 μl of an uncentrifuged control sample containing 200 μl of Tyrode's buffer, 25 μl of platelets, and 20 μl of barbital saline (usually 1,000–2,000 cpm). Percent release was calculated by dividing counts per minute in supernates by the counts per minute in the 100% control. Each sample was run in duplicate and the results were averaged.

Pneumococcus-induced platelet aggregation. Pneumococcus-induced platelet aggregation was shown by placing 100 μl of platelet-rich plasma and 10 μl of a suspension of type III pneumococci in a stirred cuvette of 6-mm I.D. at 37°C for 2 min and examining for aggregation under a phase microscope. As a control, 10 μl of barbital saline was substituted for the pneumococcal suspension. Concentration of pneumococci was 5.27 $\times$ 10^9/ml.

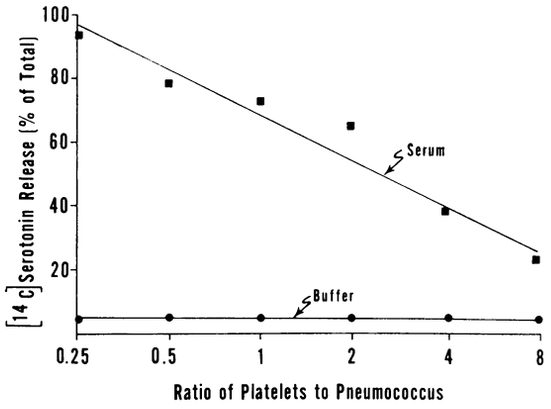

FIGURE 1 The effect of the concentration of pneumococcus on platelet release of [^{14}C]serotonin. Platelets, at a concentration of 2.4×10^7, were incubated with a decreasing concentration of pneumococcus beginning with 9.6×10^7/ml and normal human serum or buffer. The ratio of platelets to pneumococci is indicated on the horizontal axis. Diluted serum in the absence of pneumococcus did not cause release beyond that seen with buffer in the absence of pneumococcus. Type III pneumococcus was used.

Lactic dehydrogenase activity. Lactic dehydrogenase activity was determined on a DuPont automated clinical analyzer (DuPont Instruments, Wilmington, Del.).

RESULTS

Demonstration of a releasing factor in normal human plasma and serum participating in pneumococcal-induced release of [^{14}C]serotonin from human platelets. Large numbers of heat-killed pneumococci occasionally induced serotonin release in the absence of plasma or serum. However, when the number of pneumococci was reduced to approximately 2×10^8/ml or less, serum or plasma was necessary to support this release. In the following experiments a final concentration of pneumococci was chosen (usually between 7 and 9×10^7/ml) that produced no release in the absence of plasma or serum. Though a smaller number of platelets, ($2 - 6 \times 10^7$) per milliliter than pneumococci was routinely included in the reaction mixture, significant release was still seen when the pneumococcus concentration was reduced to $\frac{1}{8}$ that of the platelets (Fig. 1).

Each of 31 normal human sera tested interacted with type III pneumococci to induce the release reaction. 15 of these sera were tested with types II and VII. 14 supported release with type VII and 15 with type II. The titer of serum factor varied from individual to individual and titers were higher with type III than with the other two types (Table I). All three isolates of type III induced release equally well but only one was used for the following experiments. The method of titrating the releasing factor in normal sera is illustrated in Fig. 2. Though undiluted serum or plasma in the absence of pneumococcus usually induced moderate release, addition of pneumococcus increased the release threefold or more. It was possible to dilute most sera so that they no longer induced release beyond that seen with the diluent buffer alone (4–8%), but in the presence of pneumococcus released as much as 90% of the [^{14}C]serotonin. The ability of plasma to support release was similar to that of serum from the same individual with regard to titer. However, the absolute percentage of [^{14}C]serotonin released with undiluted

TABLE I
Titers of Releasing Factor: Comparison with IgG Concentration

Subject*	IgG	Type II titer	Type III titer	Type VII titer
	mg/100 ml			
1	750		100	
2	1,050		300	
3	1,150		100	
4	9,600		100	
5	1,100		300	
6	830		5	
7	800		10	
8	495		5	
9	3,390		5	
10	750		200	
11	920		40	
12	680		40	
13	815		20	
14	850		40	
15	1,000		40	
16	1,750		320	
17	600	20	320	20
18	1,250	10	160	10
19	925	10	160	5
20	820	80	320	40
21	1,220	20	160	10
22	1,060	40	640	40
23	780	20	320	10
24	980	80	320	80
25	1,080	20	320	10
26	860	40	320	10
27	1,020	20	160	20
28	1,360	40	320	80
29	670	40	320	20
30	600	10	160	10
31	870	2	20	0
32	<1	0	0	0

Titer is expressed as the reciprocal of the dilution giving a pneumococcus/buffer release ratio of 1.3 or greater with two different platelet preparations (see Fig. 2). No correlation between titer and IgG concentration was found (for type II $r = 0.0099$; type III $r = 0.1078$; type VII $r = 0.3371$).
* Subjects 1 through 31 were normal volunteers; subject 32 was pan immunoglobulin deficient.

plasma was sometimes less (50%) than that seen with undiluted serum.

Platelets from each of 20 normal individuals tested released at least 50% of their [^{14}C]serotonin (most released between 70 and 95%) in the presence of normal serum and type III pneumococcus.

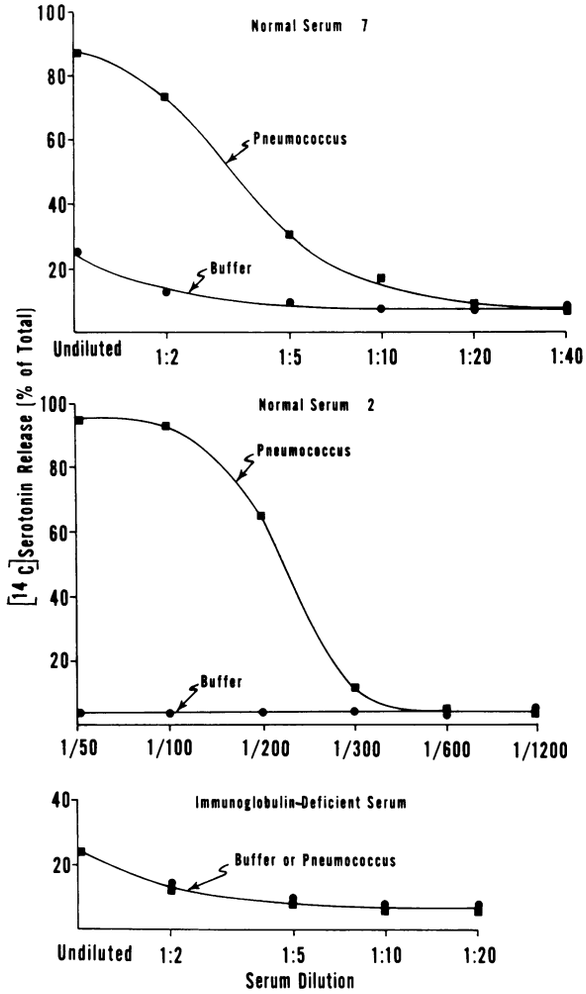

FIGURE 2 Titer of releasing factor in two normal human sera and that of an individual with severe pan immunoglobulin deficiency. Platelets, at a concentration of 2.4×10^7/ml and pneumococcus, at a concentration of 9.9×10^7/ml, or buffer, were incubated with decreasing concentrations (higher dilutions) of serum. With undiluted serum some "spontaneous" release, beyond that seen with diluent buffer alone, occurred in the absence of pneumococcus. However, with normal serum an increase in serotonin release occurred when pneumococcus was added. No such increase was seen when pneumococcus was added to immunoglobulin deficient serum. Sera, such as No. 2, with high titers of releasing factor showed no release (above that seen with diluent buffer alone) when tested at higher dilutions, unless pneumococci were added. A ratio of pneumococcal-induced release to that with buffer alone of greater than 1.3 was arbitrarily used as the end point in determining titers (1/10 for No. 7 and 1/300 for No. 2). In the experiments shown in this figure, type III pneumococcus was used.

Time course of release. Release was maximum at the end of a 1-min incubation. For convenience, however, 15-min incubations were routinely used in the following experiments.

Platelet aggregation occurred upon addition of type III pneumococcus to the platelet-rich plasma of each of six normal individuals.

Absence of platelet lysis and lack of complement requirement for release. Serotonin release appeared to be accomplished without lysis, and also without the participation of complement. After release, platelets were intact by observation on phase microscopy. In addition, no detectable leakage of lactic dehydrogenase was associated with the serotonin release. This was determined by using isolated IgG as a substitute for serum (see below). Though 70% of platelet [^{14}C]serotonin was released in the presence of type III pneumococcus and IgG (0.1 mg/ml), no lactate dehydrogenase could be found in the supernates. On freeze-thawing of the control, uncentrifuged platelet-IgG reaction mixture, however, 17 Dupont IUB U/liter were detected in the supernate.

Participation of the complement system in this release reaction appeared precluded by: (*a*) the failure of heating (56°C for 30 min), or the presence of 0.015 M EDTA, to alter the releasing capacity of serum (Table II); and (*b*) the ability of isolated IgG to support release (Table III).

Identification of the plasma/serum releasing factor as IgG. Three separate lines of evidence serve to identify the releasing factor as IgG. First, the releasing factor could be removed from diluted plasma or serum

TABLE II

Lack of Effect of Heating and EDTA on Serum Releasing Factor

Releasing factor source	Release [^{14}C]serotonin: Pneumococcus	Release [^{14}C]serotonin: Buffer	Ratio (Pn/Bu)
	%	%	
Unheated serum	86	22	3.9
Heated serum (56°, 30 min)	87	20	4.4
Serum + EDTA (0.015 M)	84	23	3.7
Buffer	5	6	0.8

Platelets were incubated with serum treated as indicated and pneumococcus or buffer. As a control, platelets were incubated with pneumococcus and/or buffer but no serum. Calcium was excluded from the platelet suspension buffer in these experiments. The ratio of release with pneumococcus to release with buffer (Pn/Bu) is indicated. Type III pneumococcus was used.

TABLE III

Releasing Capacity of Normal IgG: Comparison with IgG Fragments, Myeloma IgG, and Fibrinogen

Protein preparation	Concentration	Release [^{14}C]serotonin		Ratio (Pn/Bu)
		Pneumococcus	Buffer	
	mg/ml	%	%	
IgG	2.0	95	40.0	2.4
IgG	0.1	75	6.3	12
IgG	0.05	40	6.5	6
Fc	2.0	20.0	21	1.0
Fab	3.0	12	13	0.9
IgG_1, κ myeloma	2.0	32	30	1.1
IgG_1, λ myeloma	2.0	36	37	1.0
IgG_2, λ myeloma	2.0	20	20	1.0
IgG_3, κ myeloma	2.0	40	42	1.0
IgG_4, λ myeloma	2.0	26	32	0.8
Fibrinogen	250	6.5	7	0.9
Buffer	0	7	7	1.0

Platelets were incubated with the indicated protein (or buffer as a control) and pneumococcus or buffer. Normal and myeloma proteins at high concentrations (>2.0 mg/ml) induced significant release in the absence of pneumococcus but only normal IgG showed increased release in its presence (Pn/Bu ratio > 1.3). Normal IgG was isolated from a pool of three normal sera. Similar results were seen with IgG from a single donor or from a larger pool (American National Red Cross Cohn Fraction II). Type III pneumococcus was used.

by monospecific solid phase anti-Fab antibody (Table IV); second, serum from an individual with severe pan immunoglobulin deficiency (IgG, IgA, IgD, IgM, less than 10 μg/ml) failed to support the release reaction (Fig. 2 and Table I); and third, the release reaction was supported by preparations of immunoglobulin G isolated from normal human sera (Table III). The IgG isolated from a single individual or from pools of several individuals were equally effective in this regard. The IgG preparations gave only one line against anti-whole human serum and none of the other immunoglobulin classes were detectable on Ouchterlony double diffusion. No correlation between releasing factor activity titer and subject serum IgG concentration was demonstrated (Table I), even though the releasing factor clearly is immunoglobulin in nature. Isolated fibrinogen in a concentration of 250 mg/ml did not support release.

TABLE IV

Removal of Releasing Factor by Anti-$F(ab)_2$ Antibody

	[^{14}C]Serotonin release		Ratio (Pn/Bu)
	Pneumococcus	Buffer	
	%	%	
Plasma (1:40) + anti-$F(ab)_2$ beads	6	7	0.9
Plasma (1:40) + NR beads	73	8	9.1
Serum (1:40) + anti-$F(ab)_2$ beads	4	7	0.7
Serum (1:40) + NR beads	75	9	8.3
Buffer	3	5	0.6

Platelets were incubated with pneumococcus or buffer and with diluted plasma or serum that had been previously absorbed with an equal volume of rabbit globulin covalently linked to agarose beads (see Methods). The globulin was made either from serum monospecific for $F(ab)_2$ fragments (anti-$F(ab)_2$ beads) or from normal rabbit serum (NR beads). As a final control, platelets were incubated with buffer with and without pneumococcus. Type III pneumococcus was used.

Only the intact IgG molecule could be shown to support pneumococcal-induced release. Fc and Fab fragments prepared from isolated IgG failed to support release at concentrations 40 and 60-fold higher, respectively, than the intact parent IgG molecule (Table III).

Release was not supported to a significant degree by five myeloma IgG preparations tested at 2 mg/ml. This contrasts with the ability of a normal IgG preparation to support release at concentrations as low as 0.05 mg/ml.

DISCUSSION

Septicemia with pneumococci and other bacteria, as well as infection with viruses and fungi, may be associated with thrombocytopenia with or without associated intravascular coagulation (1-3, 7-9). The mechanism for this thrombocytopenia, however, often is not clear. Microorganisms interact with platelets in vitro in diverse ways. Some bacterial and viral products have a direct lytic effect on platelets (17, 18). Yeast cell walls (zymosan) on the other hand, require participation of the alternative complement pathway as well as fibrinogen to induce platelet release (19). One or more serum factors also participate in the interaction of several bacterial species with platelets. In this study, we have shown that for the pneumococcus and normal plasma or serum, the factor is immunoglobulin, without the participation of other plasma/serum factors. Solid phase anti-$F(ab)_2$ antibody removed the releasing factor from plasma and serum, the factor was absent from the serum of an individual with severe pan immunoglobulin deficiency (though present in the sera of all normal individuals tested), and releasing factor was present in preparations of IgG isolated from single or pooled normal sera.

Immunoglobulins have been shown to induce platelet release either when participating in immune reactions as antigen-antibody complexes (20–23) or by nonimmune mechanisms such as coating of glass or polymethylmethacrylate beads (24). Aggregated immunoglobulins of all the IgG subclasses have been shown to induce platelet release in the absence of antigen or particulate matter (25, 26). The release reaction induced by pneumococcus and immunoglobulin is typical of that seen with immune aggregates, in that it is not prevented by EDTA and, in contrast to ADP- and

epinephrine-induced release, stirring is not necessary (27, 28). The ability of type III pneumococcus to induce aggregation in platelet-rich plasma suggests that pneumococcal-induced release is not simply the result of changes occurring in the platelet during washing. Though immune complex-induced release from rabbit platelets (20-22) requires complement (29), release from human platelets does not (25, 26).

Bacteria may interact with immunoglobulin by nonimmune mechanisms as well as by the formation of antigen-antibody complexes. A cell wall component of many strains of staphylococci, known as protein A, reacts with the Fc region of several IgG subclasses (30, 31). Although some strains of pneumococcus also react with the Fc region in a nonimmune manner (32), it is unlikely that the platelet release studied here is induced by such a mechanism. Rather, several lines of evidence indicate that classical antigen-antibody complexes are responsible. First, the releasing factor titers in different normal sera do not correlate with the IgG content. Secondly, serum from a normal individual supported release induced by type III and type II pneumococcus but not type VII. Thirdly, types II and VII induced release with 15 of 15 and 14 of 15 normal sera, respectively, though these types have not shown nonimmune reactivity with the Fc portion of the immunoglobulin molecule (32). In addition, myeloma proteins at concentrations of 2 mg/ml failed to support release whereas as little as 0.05 mg/ml of normal IgG sufficed Finally, neither isolated Fc or Fab fragments of normal IgG (at 2 and 3 mg/ml, respectively) supported release, though the intact parent IgG did so at 0.05 mg/ml.

It is not known to what extent this mechanism contributes to the interaction of other microorganisms with platelets. Viral antigen-antibody complexes have been shown to induce platelet agglutination (33). Streptococcal and staphylococcal-induced platelet release and aggregation have been shown to be supported by IgG, though the characteristics of the preparation used were not specified. Unlike pneumococcal-induced release, streptococcal- and staphylococcal-induced release was also supported by fibrinogen (4).

The stimulus giving rise to the antibodies capable of interacting with pneumonococcus to promote platelet serotonin release is not known. Clinically inapparent pneumococcal infection might be responsible, as might antigens giving rise to cross-reacting antibodies (34). Regardless of their cause, their presence in all normal sera tested suggests a potential role for them in the thrombocytopenia occurring during infection with this organism.

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