COMPLEMENT-DEPENDENT PLATELET INJURY BY STAPHYLOCOCCAL PROTEIN A*

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Blood platelets are involved in a number of immune reactions and, by release of vasoactive amines and thromboplastic factor(s), may contribute to vascular injury and deposition of fibrin (1, 2). Platelet injury can be induced by bacterial endotoxins (3, 4) and immune complexes (5–7), both of which require complement, and by membrane-active bacterial toxins such as streptolysins O and S and staphylococcal alpha toxin, which act directly on the platelet membrane without the participation of complement (8).

Staphylococcal protein A (SPA),[1] a component of the cell wall of coagulase-positive staphylococci, is known to elicit a number of immune reactions such as Arthus-like reaction in rabbits and immediate skin hypersensitivity in humans (9, 10). SPA is unique among microbial products in that it interacts with the Fc portion of immunoglobulin G (IgG) rather than with the antigen-binding site on the Fab portion of the immunoglobulin molecule (11, 12), and fixes complement presumably as a result of this interaction (13). Since some of the immune reactions elicited by SPA, such as the Arthus reaction in rabbits, are thought to involve leukocytes and platelets (14), it seemed pertinent to investigate the interaction of SPA and rabbit platelets in vitro. The results of these studies indicate that (*a*) SPA does induce rabbit platelet injury, (*b*) that the reaction requires complement, and (*c*) that a sharp concentration optimum exists, larger amounts of SPA being inhibitory.

Further, evidence was obtained that SPA-induced platelet injury could be separated into two steps; first, "sensitization," an interaction of SPA with a noncomplement plasma factor and platelets, and second, amine "release" due to complement-dependent platelet injury.

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[1] *Abbreviations used in this paper:* CoF, cobra venom factor; EDTA, disodium ethylenediaminetetraacetate; 5HT, 5-hydroxytryptamine; IgG, immunoglobulin G, PPP, platelet-poor plasma; PRP, platelet-rich plasma; SPA, staphylococcal protein A.

Materials and Methods

Staphylococcal Protein A.—Crude SPA from a culture of *Staphylococcus aureus* Cowan 1 (kindly provided by Dr. Jay Cohen, Communicable Diseases Center, Atlanta, Ga.) was prepared by Jensen's heat-extraction method as described by Forsgren and Sjöquist (15). Further purification of lyophilized crude SPA was performed by ion-exchange chromatography on a diethylaminoethyl (DEAE)-Sephadex column (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) (2.5 × 45 cm) equilibrated with 0.05 M tris(hydroxymethyl)aminomethane-HCl buffer, pH 8.0. 100 mg of SPA protein dissolved in the initial buffer was applied to the column. The chromatogram was developed with an NaCl concentration gradient as illustrated in Fig. 1. Fractions were collected and analyzed for SPA activity, and those containing active material were pooled and concentrated by pressure dialysis using an Amicon UM 10 membrane filter (Amicon Corp., Lexington, Mass.). In some experiments the active material was rechromatographed under the same conditions as in the initial column separation.

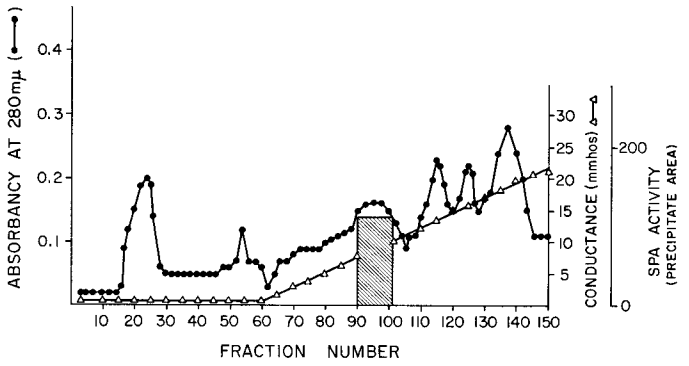

FIG. 1. Separation of SPA on DEAE-Sephadex column. Distribution of protein and SPA activity precipitating IgG (hatched bar).

Determination of SPA Activity.—Fractions were analyzed for SPA activity using the quantitative single immunodiffusion method of Mancini et al. (16) as modified by Forsgren (17). Fig. 2 (*upper panel*) demonstrates the precipitation rings caused by increasing concentrations of SPA in agar containing a standardized concentration of pooled human gamma globulin (Pentex Biochemical, Kankakee, Ill.). The precipitates were developed by staining with Buffalo black. The *lower panel* in Fig. 2 demonstrates the same assay in agar containing rabbit gamma globulin (Pentex Biochemical). Although the concentrations of the two species of gamma globulins used were similar, the diameters of the zones of precipitation and the intensity of staining of the precipitation bands was much greater with human than with rabbit gamma globulin. Tube precipitation of rabbit gamma globulins with SPA was performed following the method of Kronvall and Gewurz (18).

Disc Electrophoresis in Polyacrylamide Gel followed by Immunodiffusion.—Both crude SPA and chromatographically purified active material were analyzed by disc electrophoresis carried out by the method of Davis (19) employing a polyacrylamide concentration of 7.5% in the separation gel at pH 8.9. After electrophoresis, the gel cylinders were cut longitudinally one-half was stained for protein with Coomassie blue, and the unstained half was placed on a microscope slide and layered with 1.5% Nobel agar dissolved in Veronal buffer, pH 8.6.

After solidification of the agar surrounding the partially immersed half cylinder of polyacrylamide, a longitudinal trough cut lateral to the gel was filled with 0.1% pooled human gamma globulin (Pentex Biochemical). The preparation was incubated for 48 hr at room temperature to allow diffusion of protein from the gel and precipitation of diffusing material

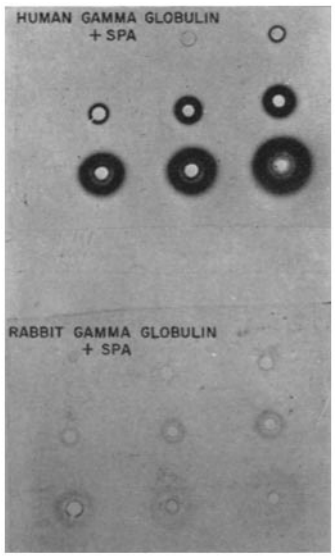

FIG. 2. Precipitation of IgG by SPA as measured by quantitative single immunodiffusion in agar. (*Upper panel*): agar contains pooled human gamma globulin in concentration 1 mg/1 ml of agar. Concentration of SPA applied to the agar: 1, 10, 50, 100, 250, 500, 1000, 1500, and 3000 μg starting with upper left well. (*Lower panel*): agar contains pooled rabbit gamma globulins. Agar was stained with Buffalo black.

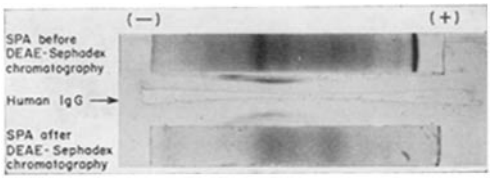

FIG. 3. Disc electrophoresis and "disc immunodiffusion" patterns of SPA. Stained polyacrylamide gel opposed to the agar at the position which was originally occupied by unstained polyacrylamide gel containing SPA.

with human gamma globulin. After incubation, the half cylinder of polyacrylamide gel was gently removed with forceps, and the slide was immersed in saline for 24 hr, distilled water for 48 hr, dried under strips of filter paper at 37°C for 24 hr, and finally stained with Buffalo black. Fig. 3 demonstrates the precipitates which developed between protein (SPA) diffusing from the polyacrylamide gel and human gamma globulin diffusing from the trough. The half cylinder of polyacrylamide which was stained for protein has been placed in the exact position of the unstained half cylinder from which diffusion took place. This combined method

of disc electrophoresis and double immunodiffusion in agar gel made it possible to identify with certainty the bands in polyacrylamide gel which contained SPA activity as demonstrated by precipitation with normal human gamma globulin. The active fraction of crude SPA demonstrated the same electrophoretic mobility in polyacrylamide as did the material purified by DEAE-Sephadex chromatography.

Other Methods.—Protein concentration was determined by the method of Lowry et al. (20). The absence of mucopeptide or teichoic acid in crude or purified SPA preparations was demonstrated by a negative reaction for *N*-acetylamino sugars after acid hydrolysis according to the method of Reissig et al. (21). Staphylocoagulase activity was assessed by the method of Tager and Hales (22) and staphylococcal alpha toxin activity by the method of Duthie (23). Neither activity was detectable in crude SPA nor the chromatographically purified fraction.

Demonstration of Platelet Injury.—Release of platelet 5-hydroxytryptamine (5HT) into plasma was utilized as the index of platelet injury. Rabbit platelet-rich plasma (PRP) was prepared by differential centrifugation (900 rpm for 10 min) of blood anticoagulated with disodium ethylenediaminetetraacetate (EDTA) (Mallinckrodt Chemical Works, St. Louis, Mo.) and heparin (Connaught Medical Research Laboratories, Westlake, Ontario) (final concentrations of 0.1% and 40 μg/ml plasma, respectively). The supernatant PRP was removed by aspiration and the EDTA then neutralized by addition of an equimolar concentration of calcium chloride. The details of PRP preparation have been previously reported (24). Aliquots of heparinized PRP were challenged with the indicated concentrations of SPA added in a volume of 0.1 ml/ml PRP and incubated at 37°C while undergoing slow rotation on an inclined disk. The reaction was terminated by addition of 10 mM EDTA after which the platelets were sedimented by high speed centrifugation at 4°C, the concentration of 5HT in the supernatant platelet-poor plasma (PPP) was determined photofluorometrically, and results were expressed as per cent of total platelet-5HT available for release.

Two-Step Experiments.—As indicated under Results, a series of experiments was undertaken to determine if SPA caused any change in platelets under conditions in which complement was inhibited. Basically, this was accomplished by exposing EDTA-PRP to SPA. After appropriate incubation (step one) the mixture was spun down, plasma containing "unbound" SPA was discarded, and platelets were washed three times in a modified Tyrode's solution according to the procedure of Ardlie et al. (25), modified by the use of EDTA rather than ethylene glycol tetraacetic acid in a final concentration of 5 mM. Subsequently, platelets were resuspended in fresh, heparinized plasma containing no EDTA and no SPA. After this reincubation (step two), the amount of 5HT released from platelets was determined. EDTA-PRP used in step one and heparinized plasma used in step two could be modified in a number of ways (see Results).

Complement Assay.—Hemolytic complement activity was determined by a modification of Yachnin's method using various dilutions of recalcified plasma in Veronal-buffered saline containing optimal concentrations of Ca, Mg, and gelatin (26). 0.5 ml of sensitized sheep red blood cells (5 x 10^8/ml) was added to 2 ml of diluted plasma and the mixture was incubated at 37°C. After 60 min of incubation, 5 ml of saline was added, the unlysed erythrocytes were sedimented, and the optical density of the supernatant was determined at 540 mμ. The results were expressed as per cent of hemolysis obtained in a control sample after lysis of the erythrocytes by freezing and thawing.

Cobra Factor.—Cobra factor (CoF) was isolated from lyophilized *Naja naja* cobra venom (Ross Allen Reptile Institute, Silver Springs, Fla.) by chromatography on DEAE-cellulose (27).

RESULTS

Release of 5HT from Platelets by SPA. Requirement for Plasma Factor(s).—Addition of SPA to platelets suspended in recalcified plasma caused release of

5HT. No release of 5HT was observed when washed platelets resuspended in buffer were exposed to SPA. Further, washed platelets exposed to SPA in buffer, rewashed, and resuspended in heparinized plasma did not release 5HT. These and similar experiments indicated that SPA did not alter platelets in the absence of plasma. The results are summarized in Table I.

TABLE I

Dependence of SPA-Induced Release of 5HT from Platelets upon Plasma-SPA Interaction

Reaction mixture	Per cent of 5HT released
	%
Platelets + plasma + SPA*	58
Platelets + Tyrode's + SPA*	3
SPA-pretreated platelets + plasma‡	3
Saline-pretreated platelets + plasma‡	9

* Platelets were washed three times in Tyrode's solution containing EDTA (see Materials and Methods) and resuspended in recalcified PPP or in Tyrode's solution containing no EDTA. SPA was added in final concentration of 100 μg/ml and system was incubated for 30 min at 37°C.

‡ SPA (100 μg/ml) or a control amount of saline was added to washed platelets in Tyrode's and incubated for 30 min at 37°C. Platelets were then sedimented, washed twice in EDTA-Tyrode's, and resuspended in heparinized PPP. This mixture of pretreated platelets and plasma was then incubated for 30 min at 37°C.

TABLE II

SPA-Platelet Interaction in Plasma with Inactivated Complement System

Reaction mixture	Per cent of 5HT released
	%
SPA + platelets + heated plasma*	0
SPA + platelets + EDTA-plasma‡	2
SPA + platelets + CoF-treated plasma§	1
SPA + platelets + untreated plasma	32

* Plasma heated to 56°C for 30 min and centrifuged to remove precipitated fibrinogen before resuspension of platelets and challenge with SPA (100 μg/ml final concentration).

‡ Plasma containing EDTA in a final concentration of 10 mM.

§ Plasma treated with cobra factor in concentration 172.5 μg/ml before resuspension of platelets and SPA challenge.

Complement Dependence of SPA-Induced 5HT Release.—Since release of platelet 5HT by SPA required plasma and since SPA is known to fix complement (13), it was necessary to determine whether complement was required for SPA-induced platelet injury. As illustrated in Table II, prior decomplementation of plasma by heat or by treatment with cobra factor prevented SPA-induced 5HT release, and the presence of EDTA in a concentration exceeding the combined concentrations of calcium and magnesium also was inhibitory.

Concentration Effect of SPA on Serotonin Release, Complement Consumption, and Precipitation of IgG.—The complement dependence of this phenomenon was further supported by experiments in which the hemolytic complement activity remaining in plasma was determined after SPA-induced 5HT release. Fig. 4 shows that release of 5HT was parallel to the consumption of complement and that both 5HT release and complement consumption were maximal within a narrow range of SPA concentration (50–100 μg), higher concentrations being inhibitory. In contrast, precipitation of IgG by SPA was directly proportional to SPA concentration throughout a wide concentration range. 5HT release and complement fixation by SPA therefore appear to be closely related phenomena.

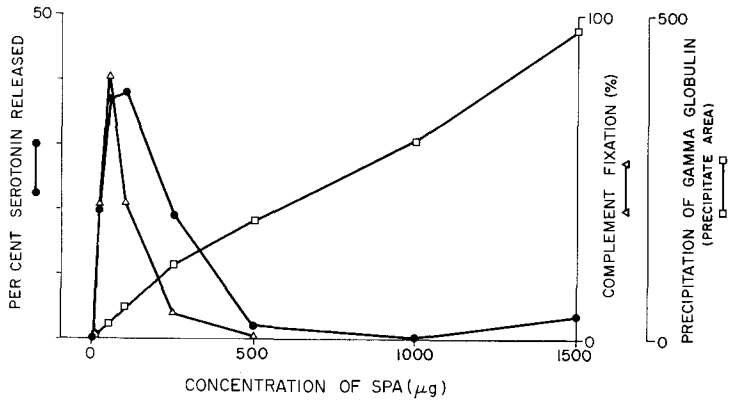

FIG. 4. Effect of SPA concentration on serotonin release, complement fixation, and precipitation of rabbit gamma globulin.

Two-Step Interaction between SPA, Plasma Factor(s), and Platelets.—The data presented above indicated that one plasma factor required for interaction of SPA and platelets was complement. Since complement fixation by SPA requires an interaction of SPA with IgG (13), it was logical to postulate that release of platelet 5HT by SPA also involved an initial SPA-IgG interaction. Indirect evidence compatible with this concept was obtained in the two-step experiments outlined schematically in Fig. 5 and described under Materials and Methods.

The essential finding of this type of experiment was that platelets exposed to SPA in EDTA-plasma (in which complement is inhibited and 5HT release is not observed) became "sensitized" or altered in such a manner that they did release 5HT when washed and then exposed to nonchelated plasma containing no SPA. As indicated earlier (Table I), similar experiments utilizing exposure of platelets to SPA in Tyrode's solution rather than in EDTA-plasma did not result in this type of sensitization to the action of complement. This was taken

as evidence that a plasma factor other than complement was involved in the initial SPA-plasma-platelet interaction.

This experimental design made it possible to distinguish conditions affecting

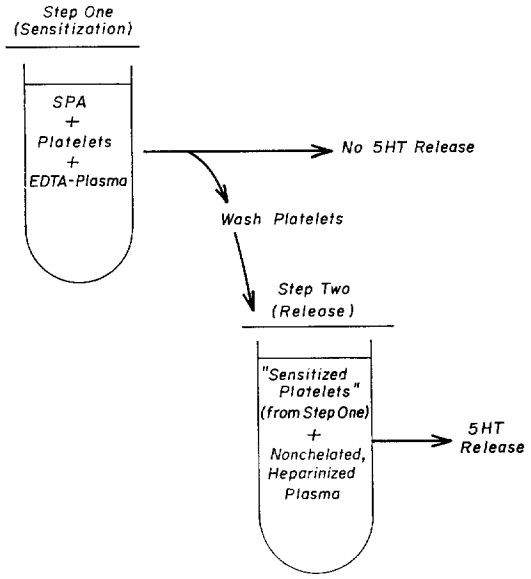

FIG. 5. Schematic representation of two-step experiments.

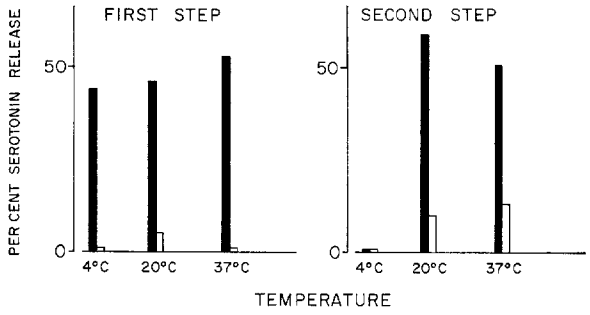

FIG. 6. Effect of temperature on two-step interaction between SPA, plasma, and platelets. Closed bars: SPA added to the system; open bars: SPA not added (control).

the interaction of SPA, noncomplement plasma factor(s), and platelets (sensitization step) from conditions affecting release of 5HT (release step). As illustrated in Fig. 6, sensitization of platelets by SPA in EDTA-plasma was not inhibited at 4°C, whereas release of 5HT from sensitized platelets did not occur at low temperature. Fig. 7 demonstrates that the sensitization phase occurred without an appreciable lag period whereas release of 5HT from sensitized

platelets was characterized by an initial lag period and progression over a 30 min period. Finally, decomplementation of plasma used in the sensitization step was not inhibitory whereas decomplementation of plasma used in the release step completely eliminated 5HT release (Fig. 8).

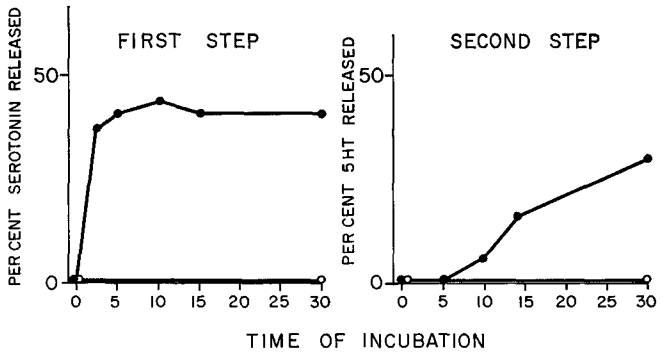

FIG. 7. Effect of time on two-step interaction between SPA, plasma, and platelets. Closed circles: SPA added to the system; open circles: SPA not added (control).

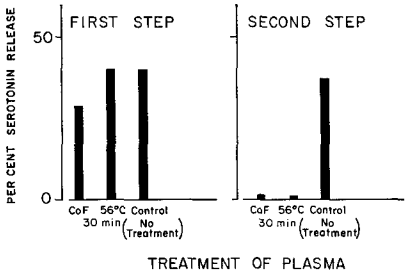

FIG. 8. Effect of decomplemented plasma on two-step interaction between SPA and platelets.

DISCUSSION

Platelet injury by staphylococcal protein A (SPA), as described herein, is a new example of cell injury by a microbial product which is not due to a direct toxic effect but rather is mediated by the complement system. It resembles in many ways platelet injury by soluble immune complexes, which also involves activation of complement, but is clearly different in that it does not depend on a classic antigen-antibody reaction.

The present experiments are submitted as evidence that the SPA-platelet interaction requires both complement and a noncomplement plasma factor. Tables I and II demonstrate that complement is necessary. Since SPA is known to cause complement fixation as a result of interaction with the Fc portion of IgG (13), and since 5HT release and complement fixation occur over the same

narrow range of SPA concentration (Fig. 4), it seemed reasonable to postulate that IgG was also involved in SPA-induced platelet injury. Demonstration of a requirement for a noncomplement plasma factor (such as IgG) could not be accomplished in single-step experiments using the complement-dependent endpoint of 5HT release. However, the two-step experiments illustrated in Figs. 5–8 indicated that platelets could be sensitized by SPA under circumstances in which complement activity was prevented. Platelets sensitized in this fashion then proceeded to release 5HT when exposed to plasma containing an intact complement system. It should be emphasized that plasma was required for sensitization even though complement was not; many experiments of the type illustrated in Table I indicated that platelets could not be sensitized by SPA unless plasma was also present. The identity of the noncomplement plasma factor required for sensitization has not been established to be IgG in these experiments, although as mentioned above there is considerable reason for believing that this is probably the case.

It is our present hypothesis that the SPA-IgG interaction exposes or activates IgG Fc determinants which then interact with receptors on the platelet membrane leading to attachment of the complex and subsequent complement fixation. Such a mechanism has been suggested to play a role in platelet injury by soluble immune complexes (28). Attempts to establish attachment of SPA-IgG complexes to the platelets using radiolabeled IgG have to date yielded inconsistent results. One possible explanation for this inconsistency has to do with the critical concentration optimum of SPA demonstrated for both complement fixation and platelet injury (Fig. 4). The concentration of SPA inducing maximal platelet injury was very close to the dose of SPA causing maximal consumption of complement but was 10 times lower than that giving maximal precipitation of IgG. This implies that full saturation of the IgG molecule with SPA (such as results in maximum SPA-IgG precipitation) will result in a loss of the ability of IgG to interact with platelets and to activate complement. Such an interpretation is concordant with observations by Kronvall and Gewurz that saturation of the Fc fragment with excess SPA prevented fixation of complement by aggregated human IgG (18). In terms of molecular weight, SPA is a relatively small molecule (mol wt 15000) (15) in comparison with the Fc fragment of rabbit IgG (mol wt 55000) (29), and its binding to the Fc fragment does not block the complement-fixing site and presumably also does not block the postulated Fc site reactive with platelet membrane receptors. It is possible that the inhibitory effect of higher concentrations of SPA may be due to polymerization of SPA on the Fc fragment of the IgG molecule. High-molecular weight polymers of SPA could block Fc sites reactive with platelet receptors on the one hand and the first component of complement on the other. Such polymers of SPA might be formed by an excess of SPA added to plasma containing platelets. This possibility is supported by the isolation of a dimeric form of SPA from SPA-IgG precipitates (15).

The concept emerging from this work may be formulated as follows: Native IgG molecules do not interact with platelets, do not activate complement, and do not cause platelet injury manifested by amine release (Fig. 9, *panel at left*). SPA in optimal concentration forms complexes with IgG which are able to interact with platelets and fix complement, leading to 5HT release (Fig. 9, *middle panel*). When higher concentrations of SPA saturate the Fc fragment and prevent it from interacting with platelets and fixing complement, no release reaction is observed (Fig. 9, *right panel*).

The mode of activation of complement by SPA-sensitized platelets is still unknown. One may infer, from analysis of the profiles of consumption of single complement components by SPA-IgG complexes (18), that the classical path-

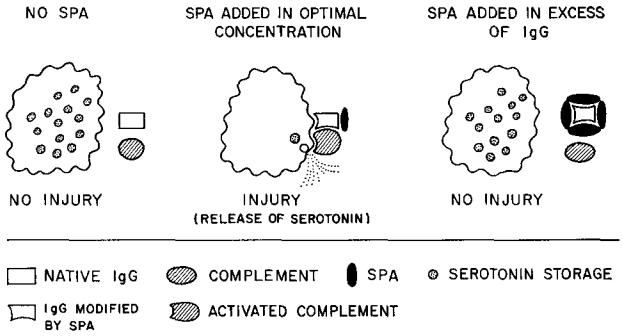

FIG. 9. A proposed concept of the complement-mediated platelet injury by the staphylococcal protein A.

way of complement fixation through C1 is involved. However, the possibility that complement is activated by SPA-sensitized platelets through the alternate pathway involving C3 activator (30) must be considered. Zimmerman and Müller-Eberhard have demonstrated that staphylococcal protein A accelerated coagulation in normal rabbit blood through a complement-mediated pathway, and they postulated that both pathways of complement activation can be involved (31). Our findings suggest that acceleration of blood clotting by SPA described by Zimmerman and Müller-Eberhard may be explained by complement-mediated platelet injury resulting in activation of thromboplastic factors (e.g., platelet factor 3) in a manner analogous to the effect of endotoxin on platelets (32).

Complement-mediated platelet damage by SPA is a newly recognized biologic property of this common component of the cell wall of coagulase-positive staphylococci. Since platelet injury and amine release play a role in vascular injury (14) and since complement-dependent factors chemotactic for polymorphonuclear leukocytes are also generated by SPA (33), the phenomenon described herein may contribute to the development of inflammatory and thromboembolic reactions to intravascular staphylococcal infection.

SUMMARY

A new example of complement-mediated platelet injury has been described. Staphylococcal protein A (SPA) causes rabbit platelet injury as manifested by release of platelet 5-hydroxytryptamine (5HT). This reaction is complement-dependent and occurs over a very small range of SPA concentration, larger amounts being inhibitory. Complement fixation by SPA demonstrates the same narrow SPA concentration requirement whereas precipitation of IgG by SPA is roughly proportional to SPA concentration over a wide concentration range. The reaction can be separated into a sensitization step which requires SPA and plasma but not complement, and a release step which does require complement.

Complement-mediated platelet damage induced by SPA is a new biologic property of this common component of the cell wall of pathogenic staphylococci which may contribute to the development of inflammatory and thromboembolic reactions complicating intravascular staphylococcal infection.

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