

EFFECTS OF BACTERIAL ENDOTOXIN ON RABBIT PLATELETS

IV. THE DIVALENT ION REQUIREMENTS OF ENDOTOXIN-INDUCED AND IMMUNOLOGICALLY INDUCED PLATELET INJURY*

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When endotoxin is injected by vein it is immediately fixed onto platelets (1). Both in vivo and in vitro studies have further shown that endotoxin causes rapid aggregation and disappearance of blood platelets, accelerates coagulation, and promotes release of several intracellular platelet factors with physiological activity (2-5). It has thus appeared likely that this platelet-endotoxin interaction may play a role in the earliest phases of removal of endotoxin from the blood stream.

While a superficially similar form of platelet aggregation and release of platelet factors is produced by thrombin, previous studies have shown that the effects of endotoxin and of thrombin on platelets differ in several important respects (6). The blood of most mammals contains antibodies to endotoxin (7). Because antigen-antibody interactions also produce platelet aggregation and release of intracellular components (8-11), the present studies explored the thesis that platelet-endotoxin interaction might be a special instance of platelet damage by antigen-antibody complexes. These experiments compare the divalent ion requirements of these two types of platelet injury. They have led to the tentative hypothesis that antigen-antibody and endotoxin injury may be identical in nature, and secondary to platelet phagocytosis.

Materials and Methods

Rabbit blood obtained by cardiac puncture was delivered directly through plastic tubing into cold sterile siliconized centrifuge tubes containing anticoagulant solutions to provide a final mixture of one part anticoagulant to nine parts blood. Platelet-rich plasma and platelet-poor plasma were prepared by centrifugation as previously described and contained from 4 to 8×10^8 platelets per milliliter (2). Platelet-rich plasma prepared directly from heparinized blood contained a much lower concentration of platelets due to spontaneous platelet clumping in this anticoagulant under the conditions employed. Accordingly, heparinized platelet-rich plasma was prepared by collection of blood in NaEDTA, preparation of platelet-rich plasma,

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addition of heparin at this point, and finally, neutralization of NaEDTA by addition of an equimolar amount of calcium chloride.

Material for immunization of rabbits was prepared by emulsifying a 5% saline solution of egg albumin (Ovalbumin 2 × crystallized, Worthington Biochemicals, Freehold, New Jersey) with an equal volume of complete Freund's adjuvant (Difco Laboratories, Inc., Detroit). This was administered in three weekly subcutaneous injections of 2 ml, each injection containing 50 mg of the antigen. Animals were not used until 2 wk after the last injection.

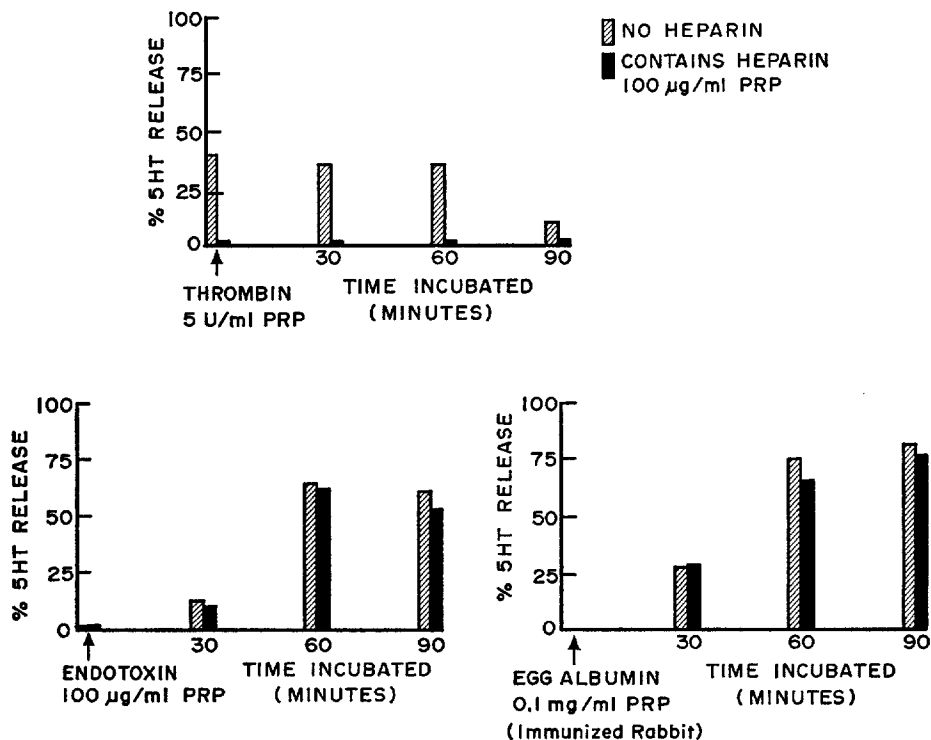


FIG. 1. Release of 5HT from platelets during the course of incubation of platelet-rich plasma (PRP) with thrombin, endotoxin, and antigen (platelet-rich plasma from immunized animals). Solid bars demonstrate the effects of heparin in these three systems.

Release of platelet 5-hydroxytryptamine (5HT) was employed as an index of platelet damage. Aliquots of platelet-rich plasma in 10 ml Erlenmeyer flasks were challenged with the appropriate additive (endotoxin, egg albumin, or saline), placed on a 12 in. diameter inclined rotary disc mixer, and rotated at 28 RPM in a 37°C bacteriologic incubator. Samples of platelet-rich plasma were removed at intervals, centrifuged to separate platelets and plasma, and the supernatant platelet-poor plasma 5HT concentration determined fluorometrically (12) using a Model A2 Photoelectric Fluorometer (Farrand Optical Co., Inc., New York). Total 5HT content of platelet-rich plasma was determined after 5 cycle freeze-thaw lysis of an aliquot removed before any experimental manipulations; this value was used in calculating the per cent of total platelet 5HT released into plasma. Challenge substances included an *Escherichia coli* endotoxin of the Boivin type (Difco, *E. coli* endotoxin, lot 0127:B8) in a 1

mg per ml saline suspension, bovine topical thrombin in a 50 NIH unit per ml saline solution, and egg albumin in a 1 mg per ml saline solution. All were added one part per nine parts platelet-rich plasma providing final concentrations of 100 μ g endotoxin, 5 NIH units thrombin, and 0.1 mg egg albumin per ml of platelet-rich plasma (PRP). Preliminary experiments demonstrated the effectiveness of these concentrations in the experimental system under study.

All glassware was siliconized (Siliclad, Clay-Adams, Inc., New York) and baked for 4 hr at 180°C to remove any contaminating bacterial pyrogen.

TABLE I
Effect of Different Anticoagulants on Release of Platelet 5HT in Platelet-Rich Plasma from Rabbits Immunized with Egg Albumin

| Anticoagulant | Challenge | Platelet 5HT released into plasma during incubation | | | |
|------------------------------|-----------|---|--------|--------|--------|
| | | 0 min | 30 min | 60 min | 90 min |
| Heparin (100 μ g per ml) | Saline | 0 | 0 | 0 | 0 |
| | Endotoxin | 0 | 33 | 88 | 85 |
| | Antigen | 0 | 30 | 72 | 75 |
| Na citrate (0.38%) | Saline | 0 | 0 | 0 | 0 |
| | Endotoxin | 0 | 53 | 71 | 76 |
| | Antigen | 0 | 0 | 0 | 0 |
| NaEDTA (0.1%) | Saline | 0 | 0 | 0 | 0 |
| | Endotoxin | 0 | 18 | 75 | 106 |
| | Antigen | 0 | 0 | 0 | 0 |
| NaEDTA (0.17%) | Saline | 0 | 0 | 0 | 0 |
| | Endotoxin | 0 | 0 | 0 | 0 |
| | Antigen | 0 | 0 | 0 | 0 |

RESULTS

Fig. 1 compares the release of 5HT from platelet-rich plasma during incubation with endotoxin or antigen (egg albumin) with that observed during incubation with thrombin. As shown, release of 5HT after challenge with endotoxin or antigen occurred after a lag period and was progressive throughout the course of the incubation. In contrast, thrombin caused an immediate release which was nonprogressive. Further, the effects of endotoxin and antigen were not inhibited in concentrations which entirely prevented release of 5HT by thrombin.

In these two respects platelet damage produced by antigen and by endotoxin were similar and quite different from platelet damage produced by thrombin. However, the influence of various anticoagulants on antigen-induced 5HT release and endotoxin-induced 5HT release differed as illustrated in Table I.

These experiments showed that endotoxin caused release of 5HT from platelet-rich plasma prepared from blood containing 0.38% sodium citrate or 0.1% sodium EDTA while antigen-antibody interaction did not. They further demonstrated another feature: when the concentration of sodium EDTA was increased very slightly, *neither* endotoxin nor antigen produced 5HT release.

| PREINCUBATION | | | | SEROTONIN RELEASE DURING INCUBATION | | | |
|----------------------|-----------------|---------|-----------------|-------------------------------------|--------|--------|--------|
| 20 Min Preincubation | | | | 0 Min | 15 Min | 30 Min | 60 Min |
| A. | Citrate (10 mM) | Antigen | Saline | 0 | 0 | 0 | 0 |
| B. | Saline | Antigen | Citrate (10 mM) | 0 | 20% | 56% | 53% |
| C. | Citrate (10 mM) | Saline | Saline | 0 | 0 | 0 | 0 |
| D. | Saline | Saline | Citrate (10 mM) | 0 | 0 | 0 | 0 |

FIG. 2. Effect of addition of citrate before and after antigen-antibody interaction on release of 5HT from subsequently added platelets. Heparinized platelet-rich plasma was prepared using blood of an immunized rabbit, divided into 4 aliquots, platelets separated from each by centrifugation and the platelet buttons set aside. Citrate was added to two aliquots (A and C) and a control amount of saline to the other two (B and D) prior to addition of egg albumin (A and B) or control saline challenge (C and D). All samples were then preincubated for 20 min following which citrate (to B and D) and saline (to A and C) were added in reverse order. Platelet buttons were then resuspended in each sample, incubation and rotation carried out, and release of 5HT measured over a further 60 min period of incubation. Platelet-rich plasma, PRP; and platelet-poor plasma, PPP.

This difference in anticoagulant requirement suggested that the mechanism of antigen-induced and endotoxin-induced platelet injury were basically different. However, this difference was not observed when antigen and platelet-poor immune plasma were allowed to interact in the absence of chelating agents. As shown in Fig. 2, preincubation of antigen in immune plasma in the absence of chelating agents resulted in the evolution of a substance, presum-

ably antigen-antibody complex, which then caused release of platelet 5HT when citrate and platelets were readded to the plasma. This was accomplished by temporarily separating the platelets from heparinized immune plasma, incubating for a 20 min period, then adding citrate, and finally resuspending platelets in the plasma. Control specimens received citrate before this period of preincubation. Thus, all aliquots were similar except for the timing of citrate addition. When further incubation of these plasmas was carried out after re-

TABLE II
Effect of Preincubation of Antigen on Release of Platelet 5HT from Immune Platelet-Rich Plasma in Different Anticoagulants*

| Anticoagulant | Challenge | Release of platelet 5HT |
|------------------------------|-------------------------------|-------------------------|
| Heparin (100 μ g per ml) | Saline | 0 |
| | Endotoxin | + |
| | Antigen | + |
| | <i>Preincubated antigen</i> ‡ | + |
| Na citrate (21.5 mM)§ | Saline | 0 |
| | Endotoxin | + |
| | Antigen | 0 |
| | <i>Preincubated antigen</i> ‡ | + |
| NaEDTA (4.5 mM)§ | Saline | 0 |
| | Endotoxin | + |
| | Antigen | 0 |
| | <i>Preincubated antigen</i> ‡ | + |
| NaEDTA (7.5 mM)§ | Saline | 0 |
| | Endotoxin | 0 |
| | Antigen | 0 |
| | <i>Preincubated antigen</i> ‡ | 0 |

* Egg albumin in platelet-rich plasma obtained from immunized rabbits.

‡ Egg albumin preincubated with heparinized platelet-poor plasma obtained from an immunized rabbit prior to additional citrate or NaEDTA and resuspension of platelets.

§ Millimolarity of chelating agent in plasma.

suspension of platelets, tubes in which antigen and antibody were preincubated in the temporary absence of citrate had come to resemble those which contained endotoxin. In such systems, release of platelet 5HT then took place in the presence of citrate (Table II). Fig. 3 shows that the capacity of antigen to release 5HT in the presence of citrate was acquired within 10 min preincubation, was maximum at 60 min, and deteriorated slightly with longer preincubation.

In considering the reasons for the differences observed, the relative concen-

trations of ionized divalent cations in the solutions utilized were examined. Table III compares the ionized divalent cation concentrations of the anticoagulant solutions used. Normal rabbit plasma magnesium and calcium concentrations have been found to average 1.01 mM (0.84 to 1.22 mM) and 3.84

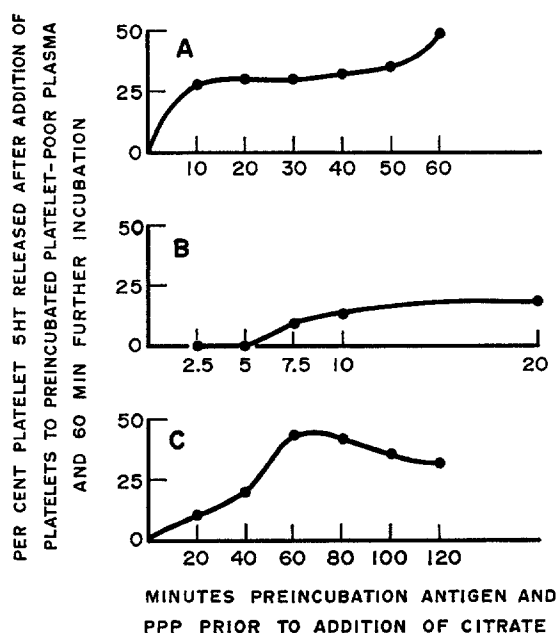


FIG. 3. Development of citrate resistant 5HT releasing capacity during preincubation of antigen in heparinized platelet-poor plasma (PPP) from immunized rabbit. Aliquots of heparinized platelet-rich plasma were prepared, the platelets separated by centrifugation, and antigen (0.1 mg egg albumin per ml plasma) added to each tube. In the experiment depicted in A, citrate (10 mM) was added to the first tube before antigen challenge, to the second after 10 min preincubation, to the third after 20 min, and in like manner to successive samples at 10-min intervals throughout a 60 min period of preincubation. Saline was added to the last sample as a control. After preincubation, platelets were resuspended in all aliquots and release of 5HT determined after 60 min further incubation. Per cent 5HT release is represented on the ordinate, time of preincubation before addition of citrate on the abscissa. The experiment depicted in B focuses on the development of 5HT-releasing capacity during the first 10 min of preincubation. The experiment depicted in C demonstrates the effects of more extended preincubation.

mM (2.6 to 5.0 mM) respectively (13). Since heparin is not a chelating agent, these concentrations can be presumed to be present in heparinized plasma. The plasma molar concentration of citrate in blood anticoagulated with 0.38% sodium citrate exceeds the combined plasma concentrations of calcium and magnesium. However, citrate is a relatively weak ligand (14), and one can

calculate that citrated plasma contains approximately 0.1 mM concentrations of both ionized calcium and magnesium. In contrast, sodium EDTA binds both calcium and magnesium much more avidly (14). When present in plasma in amounts exceeding the combined concentrations of calcium and magnesium, virtually no free divalent cation remains. The commonly used concentration of 0.1% EDTA represents a special case: the molar plasma sodium EDTA concentration of 0.1% sodium EDTA blood (assuming an hematocrit of 40%) is slightly greater than the plasma concentration of calcium ion but less than the combined plasma concentrations of calcium plus magnesium ion. This excess of unbound divalent cation results in quite different concentrations of both free magnesium and free calcium ion; with respect to concentration of

TABLE III
Characteristics of Anticoagulant Solutions Employed

| | Heparin | Na citrate (0.38%) | NaEDTA (0.1%) | NaEDTA (0.17%) |
|------------------------------|-------------------------|-----------------------|------------------|-------------------|
| Concentration in whole blood | 30-100 $\mu\text{g/ml}$ | 12.9 mM | 2.7 mM | 4.5 mM |
| Concentration in plasma* | 50-166 $\mu\text{g/ml}$ | 21.5 mM | 4.5 mM | 7.5 mM |
| pK calcium† | — | 3.22 | 10.59 | 10.59 |
| Plasma ionized calcium§ | 3.8 mM | 0.1 mM | 0.01 mM | <0.000001 mM |
| pK magnesium† | — | 3.20 | 8.69 | 8.69 |
| Plasma ionized magnesium§ | 1.2 mM | 0.1 mM | 0.6 mM | <0.000001 mM |

* Plasma concentration = concentration in whole blood \times 100/(100-hematocrit) using hematocrit = 40%.

† Reference 14.

§ Approximate values calculated from † and ||.

|| Mean values from reference 13.

nonchelated divalent cations, 0.1% sodium EDTA blood resembles citrated blood much more closely than it resembles 0.17% sodium EDTA blood.

By comparing Tables II and III the divalent ion requirements of these three types of platelet injury can be partially defined. Interaction of antigen and antibody resulting in release of platelet 5HT requires more divalent cation than is furnished in citrated plasma or in plasma prepared from 0.1% sodium EDTA blood. These plasmas however do furnish sufficient divalent cation to support release of platelet 5HT by endotoxin. Preincubation of antigen in immune heparinized plasma converts the system to one which then resembles endotoxin in its reactivity in either citrated or 0.1% sodium EDTA plasma. When larger concentrations of sodium EDTA are used the resultant plasma does not contain sufficient free divalent cation to support any of these reactions.

DISCUSSION

The present studies confirm the fact that the interaction of endotoxin and platelets has a specific divalent ion requirement. Parallel studies using glycogen have demonstrated that platelet damage induced by this particulate agent has identical divalent ion requirements. Moreover neither of these two types of platelet damage are inhibited by heparin and both have similar lag periods. For reasons to be discussed below, we have come to believe that these reactions may represent platelet phagocytosis of particulate matter.

Platelet damage produced by addition of antigen to immune platelet-rich plasma requires considerably more free divalent cation than necessary for the platelet-endotoxin interaction. However, preincubation of antigen in immune plasma containing no chelating agent converts the system to one which then demonstrates the same divalent ion requirements as the endotoxin-platelet interaction. It seems reasonable to suggest that during this period of preincubation, antigen and antibody interact with complement to evolve antigen-antibody complexes which then interact as particles with platelets. Siqueira and Nelson have in similar fashion defined two phases of antigen-antibody-induced platelet injury (9). The first phase, during which antigen and antibody were incubated together, was inhibited by citrate and other chelating agents. The second phase, which occurred during incubation of this mixture with platelets, was found to be active in the presence of citrate. These authors presented considerable evidence suggesting that the heat-labile plasma factors required for the second phase of this interaction are not identical with complement. In contrast, Gocke has clearly demonstrated the participation of complement in the production of platelet injury by *soluble* antigen and antibody (15). Coupling these two studies with the present data one can construct the following thesis: antigen, antibody, and complement interact in the presence of physiologic concentrations of ionized divalent cation to form a complex which then can react with platelets in a fashion not involving further participation of complement and requiring definite but much smaller concentrations of divalent cation such as are provided in citrated plasma. The reaction between endotoxin and platelets requires heat-labile plasma factors which in like manner do not appear to be complement (16, 17). Current studies are attempting to compare these factors with those required for the interaction of preformed antigen-antibody complexes with platelets.

Recent studies by Movat and associates offer some support for the thesis that both endotoxin and antigen-antibody complexes behave as particles and that platelet injury produced by these substances is secondary to phagocytosis (18, 19). These authors have shown that platelets ingest latex particles, ferritin-antiferritin complexes, and other particulate materials. These platelet-particle interactions were independent of complement, produced release of platelet histamine and 5HT, and resulted in platelet degranulation. Since 5HT is principally granule-associated in the platelet (20, 21), it can be perhaps regarded as a component of platelet lysosomes. Thus in many respects platelet-particle interactions result in a series of events analogous to that known to occur during the course of leukocyte phagocytosis (22).

The question of the physiologic importance of platelet phagocytosis in infectious

diseases merits examination. A series of clinical observations suggests that the platelet may be involved in a variety of septicemic states. Diseases such as subacute bacterial endocarditis (23), malaria (24), Rocky Mountain spotted fever (25), typhoid fever (26), Gram-negative septicemias (27), Dengue fever (28), and meningococcal infections (29) have been noted to be associated with thrombocytopenia. Consequences of overwhelming sepsis such as renal cortical necrosis and adrenal hemorrhagic thrombosis of the Waterhouse-Friderichsen type have been attributed to intravascular coagulation (30-33) and may well evolve in part at least from platelet injury.

In like manner many of the effects of immunologic injury seem to implicate platelets. Platelet aggregation and release of vaso-active amines are characteristic features of anaphylaxis. Recent analyses of both experimental and clinical acute glomerular nephritis suggest that the kidneys may be injured in these conditions by fibrin generated elsewhere in the circulation (34, 35), possibly as a result of particle-platelet or immune complex-platelet interactions.

An hypothesis integrating these various considerations views the platelet as a participant in the earliest phases of blood stream clearance of a variety of particulate and macromolecular materials (36). It seems reasonable from this point of view that some of the manifestations of systemic infection may be consequences of the normal operation of this clearance mechanism in circumstances in which the amount of material to be removed is quite large. Within this framework, some manifestations of immunologic injury can be regarded as consequences of a special form of particle clearance requiring the interaction of antigen, antibody, and complement so that the foreign protein may be recognized and treated as a particle.

SUMMARY

The divalent ion requirements of rabbit platelet injury by endotoxin have been defined by the use of various anticoagulant solutions and have been compared to the divalent ion requirements of platelet injury produced by addition of antigen to immune platelet-rich plasma. The endotoxin-platelet interaction takes place in citrated blood. Platelet damage by antigen is inhibited by citrate, but preincubation of antigen and immune platelet-poor plasma in the absence of citrate results in a substance, presumably antigen-antibody complement complex, which then does injure platelets in the presence of citrate. Neither endotoxin nor preincubated antigen injures platelets in the presence of sodium EDTA in concentrations sufficient to interact with all divalent cations present in plasma.

These observations have been interpreted by viewing the platelet-endotoxin interaction as a consequence of platelet phagocytosis of endotoxin, a reaction not requiring complement but requiring definite small concentrations of divalent cations. The interaction of antigen and platelets is regarded as a two phase reaction, the first requiring the participation of complement and concentrations

of divalent cation larger than those provided in citrated plasma, the second requiring smaller concentrations of divalent cation, no further participation of complement, and active in citrated plasma. This second phase is regarded as representing platelet phagocytosis of immune complexes.

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