Comparative Study of Blood Clotting Factors in Anaphylactic and Primary and Secondary Endotoxin Shock

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ABSTRACT

Cinephotomicrography shows emboli are formed in the lung during stages of anaphylactic and endotoxin shock and platelet counts are decreased. Rabbits in anaphylactic shock had a 50 percent mortality while 100 percent mortality was associated with endotoxin shock. During the terminal phase, the platelet count recovers in anaphylactic shock but not in endotoxin shock. This suggests a difference in the emboli formed during these two forms of shock. This study was directed at determining what might be the difference.

Periodic measurement of electrocardiographic tracings, fibrinogen, factor VIII and fibrin degradation products (FDP) were made using standard equipment and test kits.

The results of the study showed no changes in any of the measured clotting factors during anaphylactic shock. However, in endotoxin shock FDP appeared after seven to 10 hours, fibrinogen levels decreased from 221 mg per dl to 85 mg per dl and factor VIII time increased significantly.

From these results, it is concluded that platelet aggregation in anaphylactic shock does not involve fibrin deposition, whereas in endotoxin shock platelet aggregation and fibrin deposition are both a factor in the formation of the emboli, possibly explaining the irreversible aggregation observed in terminal endotoxin shock.

Introduction

In vivo cinephotomicrography has visually demonstrated emboli lodging in the microcirculation during endotoxin and anaphylactic shock. With this technique, however, it is not possible to determine visually whether these emboli are composed of platelets alone or a combination of platelets and fibrin. Both anaphylactic and endotoxin shock show similar patterns in platelet decrease, venous and arterial pressure changes, and electrocardiogram tracings. A comparison of clotting factors in the blood may point out differences that could be ad-
vantageous to a better understanding of these two syndromes.

It was suggested by us in an earlier study\textsuperscript{16} that the severity of anaphylaxis and endotoxin shock may not only depend on humoral factors and the degree of platelet drop, but also the involvement of the intrinsic clotting system in the deposition of fibrin. Detection of plasma clotting factor changes during these critical periods of anaphylaxis and endotoxin shock might verify the involvement of fibrin in this form of embolization.

Two phases of platelet aggregation with resultant thrombopenia are known to take place in endotoxin shock.\textsuperscript{32} After the first phase, the initial platelet count never completely returns to normal and it is suspected that some irreversible platelet aggregation occurred, possibly accompanied by changes in other clotting factors. This assumption could be verified by analysis of selected clotting factors during this time.

The second platelet decrease in endotoxin shock, by all indications, appears to be irreversible. A study of the intrinsic clotting system in both anaphylactic and endotoxin shock, when the platelet count diminishes, might show the type of platelet aggregation taking place and help determine the type of treatment to be most beneficial at the various stages of shock. A preliminary report of this work has been published.\textsuperscript{6}

Materials and Methods

New Zealand albino rabbits were used in all of the experiments. In the anaphylactic shock study, they were sensitized with 2 ml of human serum injected slowly into the marginal ear vein on two consecutive days. Shock was induced 12 days after the initial sensitizing dose. The rabbits were anesthetized with 30 mg per kg of body weight of sodium pentobarbital. The shock dose, consisting of 0.5 ml of human serum diluted to 1 ml with isotonic saline, was slowly injected into the marginal ear vein over a 30 second time period.

Endotoxin shock was induced using 1 mg of \textit{Escherichia coli} (0127.B8) lipopolysaccharide per kg of rabbit weight dissolved in 3 ml of saline. The endotoxin was injected into the marginal ear vein over a period of 90 seconds.

A 16 gauge catheter with a three way stopcock was inserted into the left carotid artery of each rabbit. One lead was attached to an anaroid manometer to monitor arterial pressure. The second opening of the stopcock was used to draw blood. Just before a test sample was drawn, 2 ml was first drawn into slightly heparinized saline and then immediately after, the test sample was drawn using a different syringe, and the diluted saline blood was then returned to the animal. This procedure insured a fresh sample each time. The amount of heparin used in the saline and for flushing the catheter to prevent the blood from clotting was 2.5 units per ml.

Small areas on the legs of the rabbits were shaved and the leads were attached to these areas using alligator clamps for the electrocardiogram tracings.* Blood for platelet counts was collected directly from the stopcock.† Direct platelet counts were performed.‡

Fibrinogen and factor VIII determinations were performed using 2.7 ml of blood delivered to a tube containing 0.3 ml of 3.2 percent (w/v) sodium citrate in deionized water. The tube was gently mixed and centrifuged at 2000 \texttimes g for 10 minutes. The plasma layer was decanted from the cells, and the fibrinogen level was determined as described by Ware et al.\textsuperscript{35} Factor VIII was determined as described by Langdell et al.\textsuperscript{20} Fibrin

\* Sanborn 500 Viso Cardiette, Sanborn.
† Becton-Dickinson Unopette 5855, Becton-Dickinson, Rutherford, NJ 07073.
‡ American Optical hemocytometer, American Optical Corporation, Buffalo, NY 14240.
degradation products (FDP) were determined.

Results

Anaphylactic Shock

The most critical period during an anaphylactic reaction occurred within three to seven minutes after the shocking dose of antigen was administered. During this time the platelet count drastically dropped, accompanied by a fall in arterial pressure (table I).

In all cases when the platelet count declined, the arterial pressure fell and as the platelet count approached normal, the pressure rose. The analysis for FDP was negative for each rabbit at the critical time of five minutes after the antigen was injected, and again at 60 minutes. This denotes that there was no substantial amount of FDP circulating in the blood, suggesting strongly that no fibrin deposition occurred. This was further verified in that no significant decrease in fibrinogen occurred, as it would have during a clotting process. Factor VIII time varied slightly at five minutes and was slightly elevated at 60 minutes.

The results of the electrocardiograms showed an erratic pattern after the antigen was injected and the platelet count decreased. Following the critical period of seven minutes, the platelets began to deaggregate and the electrocardiogram again appeared normal. In one animal, in which death resulted, the electrocardiogram never returned to normal and showed a suppressed ST segment and eventual heart block.

For the animal that expired, no platelets could be detected in the blood after six minutes. The fibrinogen level did not drop but was slightly elevated (153 mg per dl to 160 mg per dl) and analysis for FDP was negative, although the factor VIII time was slightly increased (46 sec to 55 sec). In this case, as well as in the others, one would assume that fibrin plugging did not have a major role in this syndrome and that platelet aggregation occurred without significant coagulant release from the platelets. The procedure for the control was exactly the same using a nonsensitized rabbit and the results showed no drop in the platelet count or arterial pressure, no changes in the electrocardiogram and no significant change in the clotting components.

Endotoxin Shock

The same procedure and blood drawing times of the endotoxin experiments were used on two control animals; however, isotonic saline was substituted for the injection of endotoxin. In control 1, the fibrinogen level increased after 16 hours; in control 2, it remained about the same. In both control animals, factor VIII times did not vary and the platelet count did not drop during the 16 hour period. Analyses for FDP were all negative.

The animals in endotoxin shock had quite different patterns from the two controls. As previously reported, there appear to be three stages occurring in endotoxin shock. The results are shown in table II. A severe initial platelet drop

<table>
<thead>
<tr>
<th>Time (Minutes)</th>
<th>0</th>
<th>5</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arterial pressure (mm Hg)</td>
<td>121.8 ± 5.1</td>
<td>82.8 ± 20.6</td>
<td>105.5 ± 16.2</td>
</tr>
<tr>
<td>Platelet count (x10^3)</td>
<td>221.5 ± 169</td>
<td>35.0 ± 31.1</td>
<td>105.5 ± 88.5</td>
</tr>
<tr>
<td>Fibrin split products</td>
<td>Negative†</td>
<td>Negative†</td>
<td>Negative†</td>
</tr>
<tr>
<td>Fibrinogen (mg per dl)</td>
<td>277.3 ± 95.3</td>
<td>285.7 ± 88.1</td>
<td>261.9 ± 99.6</td>
</tr>
<tr>
<td>Factor VIII (seconds)</td>
<td>37.6 ± 8.4</td>
<td>36.5 ± 10.8</td>
<td>41.6 ± 8.2</td>
</tr>
</tbody>
</table>

*All values are the mean S.D. (n = 8)
†All eight rabbits gave negative tests

§ Thrombo-Wellcotest kit procedure, Burroughs Wellcome Co., Research Triangle Park, NC 27709.
Summary Of Changes In Coagulation Factors During Endotoxin Shock In Rabbits*

<table>
<thead>
<tr>
<th>Time (Hours)</th>
<th>0</th>
<th>0.084</th>
<th>1</th>
<th>7 - 10</th>
<th>(Death) 14 - 36</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arterial pressure (mm Hg)</td>
<td>119.8 ± 8.1</td>
<td>84.1 ± 18.3</td>
<td>104.2 ± 9.9</td>
<td>98.4 ± 28.1</td>
<td>22.2 ± 6.7</td>
</tr>
<tr>
<td>Platelet count (x10^3)</td>
<td>346.5 ± 106.1</td>
<td>10.4 ± 6.1</td>
<td>139.0 ± 44.5</td>
<td>94.9 ± 37.9</td>
<td>61.5 ± 47.6</td>
</tr>
<tr>
<td>Fibrin split products</td>
<td>Negative†</td>
<td>Negative†</td>
<td>Negative†</td>
<td>Positive‡</td>
<td>Positive§</td>
</tr>
<tr>
<td>Fibrinogen (mg per dl)</td>
<td>221.1 ± 100.6</td>
<td>218.9 ± 66.1</td>
<td>229.2 ± 58.5</td>
<td>121.4 ± 19.2</td>
<td>85.0 ± 49.3</td>
</tr>
<tr>
<td>Factor VIII (seconds)</td>
<td>35.9 ± 6.9</td>
<td>39.2 ± 8.3</td>
<td>40.5 ± 9.8</td>
<td>52.5 ± 9.8</td>
<td>60.4 ± 12.5</td>
</tr>
</tbody>
</table>

*All values are the mean ± S.D. (n = 9)
†All nine rabbits gave negative tests
‡Seven of nine rabbits gave positive tests
§All nine rabbits gave positive tests

occurred at five minutes after the endotoxin injection concomitant with an arterial pressure drop (Phase I) followed by a partial recovery with a rise in the platelet count and return to a near normal pressure (Phase II). Gradually the platelet counts again decreased with a drop in arterial pressure during the last several hours (Phase III). The electrocardiograms, shown in our earlier work, clearly distinguished the three phases. At five minutes, an abnormal pattern similar to that seen in anaphylaxis appeared, suggesting a similarity between the two shock syndromes. Partial recovery then became apparent and the terminal stage displayed a depressed ST wave similar to that seen in fatal anaphylaxis. Finally, heart block develops eventually leading to the death of the animals. The blood clotting assays (table II) show that the fibrinogen levels remain essentially unchanged at five minutes when the arterial pressure and platelet counts were low. There was no significant change in the factor VIII times, and all the assays for FDP were negative. From these data, it is assumed that the initial platelet aggregation occurred without any significant liberation of pro-coagulant material from the platelets.

During the secondary platelet aggregation, in Phase III of endotoxin shock, all three coagulation categories confirmed clotting. There was a significant drop in the plasma fibrinogen level, the average drop of all nine determinations being 136 mg per dl in 20 hours. With fibrin being deposited in the microcirculation and enzymatically acted upon by plasma, the FDP level would be expected to increase. This was confirmed in all cases where the third stage gave a positive test for FDP. Factor VIII times rose significantly demonstrating the utilization of this factor. With these results, there appears little doubt that secondary platelet aggregation in endotoxin shock is accompanied by fibrin deposits.

**Discussion**

There were no results in the anaphylactic shock experiments or in the initial endotoxin shock phase that indicated that the intrinsic clotting system was involved to any substantial extent. Indications are that platelet aggregation alone may be the major contributing factor for the emboli observed with cinephotomicrography in these two syndromes. Considering neither was involved in fibrin degradation, there remains the difficult task of accounting for the fatalities of allergic shock since initial endotoxin and anaphylactic shock have similar patterns in platelet drop, arterial pressure changes, and electrocardiograms.

The humoral influence may be the possible combining source for the ob-
served deaths in anaphylaxis. Okpako\textsuperscript{28} found that sensitized isolated guinea pig lungs perfused with tyrode solution responded to the injection of antigen. Histamine appeared in the effluent along with a simultaneous bronchoconstriction. The peak release of histamine occurred at three minutes and then declined rapidly. More than 90 percent of the total histamine released was within the first five minutes. This time period coincides with the critical interval when platelet aggregation takes place and death may result. Histamine has been shown to be released \textit{in vitro} from human lung tissue and thought to be a major mediator of anaphylaxis in man.\textsuperscript{29,33} In animal studies,\textsuperscript{5} other humoral factors such as bradykinin were shown to be an active vasodilator and capable of increasing capillary permeability and may be important in animal physiology and this current study. However, human examinations have not demonstrated its involvement in anaphylaxis.\textsuperscript{1} Likewise, serotonin has been implicated in animal anaphylaxis\textsuperscript{7} but not in man.\textsuperscript{1} Our administration of antigen was quick and direct, like the perfused lung, and resulted in an immediate and total response within five minutes. Treatment within such a short time would be difficult. In most clinical situations, the onset of symptoms is slower where adequate preventative treatment is feasible. Parenteral injection of antigen produces symptoms up to 60 minutes later and oral ingestion of antigen could cause symptoms hours later.\textsuperscript{19}

The initial treating phase of endotoxin shock may also fall within this category. It has been shown that endotoxin increases blood histamine levels in dogs.\textsuperscript{36} Hook et al\textsuperscript{12} demonstrated that endotoxin releases histamine from mass cells of hamsters. Dogs pre-treated with a histamine liberator, first depleting histamine stores, showed little improvement and then only in the early phase of endotoxin shock.\textsuperscript{10} However, Zeppa\textsuperscript{38} could not find an increase of histamine after endotoxin administration in dogs. In our experiments, no deaths were witnessed in the initial phase of endotoxin shock. If histamine was involved in the early phase of endotoxin shock, its involvement must be minor when compared to anaphylactic shock where histamine is felt to be a major pathologic substance. The use of an antihistamine drug in the early treatment of endotoxin shock is not well founded. The use of a platelet anti-aggregating drug appears to be extremely important to the final outcome of endotoxin shock since 50 percent of the platelets remained aggregated in the initial shock phase.

The present work suggests that anticoagulant therapy, such as heparin, could also be effective in endotoxin shock but probably not as effective in anaphylactic shock since fibrin was not involved. Heparin alone was shown not to prevent platelets from aggregating, but when injected together with endotoxin did prevent fibrin from being deposited in the kidney glomeruli of rabbits.\textsuperscript{2} Heparin was demonstrated not to prevent endotoxin death in rabbits but did increase the percent survival in rats.\textsuperscript{21} Streptokinase also prevented fibrin deposits in the glomeruli during endotoxin shock but the platelet count still dropped and the animals died within 10 hours.\textsuperscript{2}

The failure of many present drugs to prevent primary platelet aggregation by adenosine diphosphate (ADP) may be a major hindrance in the management of anaphylactic and endotoxin shock. Platelet aggregation alone, without fibrin involvement, may be adequate enough to cause an irreversible state of shock as demonstrated in heparin and streptokinase treated animals.\textsuperscript{2,21} Until platelet aggregation can be completely controlled, this problem will remain a complex and difficult one to solve.

\textit{In vitro} investigations have shown aspirin, as well as other anti-inflammatory
drugs such as phenylbutazone and sulfinpyrazone, does not inhibit primary aggregation with ADP but prevents the formation of the secondary aggregation curve and inhibit and release mechanisms. Some investigators believe that all platelet aggregation occurs through stimulated release of ADP from platelets. Previous studies from our laboratory have shown that rabbits with initial high plasma ADP levels had a higher death rate in anaphylaxis and a shorter survival time in endotoxin shock when compared to animals with initial low plasma ADP levels. Thus, the usefulness of anti-inflammatory drugs in anaphylaxis may be doubtful because of the inability of anti-inflammatory drugs to prevent initial ADP aggregation and because the release mechanism does not seem to be involved in this syndrome. This study has shown fibrin deposition to be decisively involved in terminal endotoxin shock and possibly activated by the irreversible platelet aggregation and release mechanism. In this case, prevention of platelet release with these drugs may be beneficial in preventing fibrin deposits and possibly the secondary platelet aggregation phase from occurring. Contrary to the in vitro ADP mechanism, aspirin also appears to improve the initial phase of endotoxin shock. The pre-treatment of dogs with acetylsalicylic acid prevented the fall in mean systemic arterial pressure and the associated portal hypertension following the injection of endotoxin.

It has been suggested that platelet aggregation by antigen-antibody complexes is induced by releasing ADP during phagocytosis of the complexes. This may represent an important pathogenetic mechanism in anaphylaxis. Other workers have shown that antigen-antibody complexes affected platelets in much the same way as thrombin. The hypothesis has been made that antigen-antibody and endotoxin injury may be identical in nature and secondary to platelet phagocytosis. That particulate matter causes in vivo and in vitro platelet aggregation has been known for a long time. Studies with ellagic acid showed that Hageman factor was activated and platelets agglutinated, causing the release of platelet factors into the plasma. These workers suggest that Hageman factor activation by endotoxin need not be a direct effect but may be induced by an indirect effect mediated by changes in the animal organism.

The release of platelet Factor 3 during irreversible platelet aggregation has been attributed to being a major contributor in activating the coagulation system. Around 50 percent of the platelets in the initial phase of endotoxin shock in our series appeared to aggregate irreversibly but no clotting changes were recorded at that time and in some animals clotting changes did not appear for hours. It has been found that the infusion of purified platelet Factor 3, with an activity three times that usually available in normal rabbits, failed to initiate intravascular coagulation. This suggests other factors may also be responsible for the intravascular coagulation (theory of pluricausality). The initial insult of platelet aggregation without de-aggregation could set off a chain of ischemic events causing injury to cellular tissues which may be conducive to the coagulation process. McGrath and Stewart demonstrated severely damaged endothelial cells one hour after endotoxin administration. Hemolysis of red cells may activate the fibrinolytic enzyme system. Red cell sequestration was found to occur in baboons by measuring Cr-labelled red cells one hour after the intravenous injection of a lethal dose of live E. coli organisms. These cells possibly were trapped in an ischemic environment detrimental to their membrane stability. Lysosomes were found to be released into the surrounding fluids after the injection of endotoxin and could be instrumental in initiating clotting. Glucocorticoids have
been reported to be successful in the treatment of shock and shown to inhibit lysosomal enzyme release\textsuperscript{15} by preserving lysosomal membranes.\textsuperscript{18} It has been shown that platelet-endotoxin interaction is both dose and temperature dependent and is similar to the effects of antigen-antibody union in the presence of platelets.\textsuperscript{13} However, these workers failed to implicate participation of coagulation factors other than Factor 3.

Acknowledgments

The authors wish to express their appreciation to Carol Hillman for valuable technical assistance.

References


