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Platelet Aggregation in Rabbits Made Tolerant to Endotoxin

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Endotoxin may cause abnormal deposition of platelet-endotoxin aggregates, and this event could have damaging effects. We compared the aggregation characteristics of platelets from rabbits made tolerant to the lethal effects of endotoxin with those of platelets from normal rabbits. Platelets from tolerant rabbits aggregated more rapidly (>90 s faster) in the presence of endotoxin than did platelets from nontolerant animals. Furthermore, platelets from tolerant animals aggregated reversibly. These characteristics of platelets from tolerant animals are due to humoral factors in the plasma, because 1:1 dilution of normal platelet-rich plasma with plasma from tolerant rabbits caused the normal platelets to behave like those from tolerant animals. Survival after challenge with lethal quantities of endotoxin was enhanced in tolerant rabbits; this may be due to promotion of more efficient removal of endotoxin-platelet complexes from the blood by the reticuloendothelial system.

Platelets from rabbits and many other nonprimates undergo progressive aggregation in the presence of endotoxin, and this process seems to be important in some species in the genesis of endotoxin-induced shock. Lethal doses of endotoxin induce accumulation of platelet aggregates in capillary beds of major organs, and reduction of this process through biochemical treatments eliminates fatal consequences (5).

Platelet aggregation enhances particle transport to the reticuloendothelial (RE) system. Substances that affect platelet number or function have a profound influence on the clearance rate of intravenously injected carbon (4, 15, 17) or endotoxin (3). Platelet-particle complexes must pass the capillary network of non-RE organs to reach RE tissues, and this process depends on the maintenance of a balance between aggregation and disaggregation of platelets (17). Once formed, platelet aggregates release mediators that enhance vascular permeability, damage endothelium, and induce leukocyte accumulation (11, 18).

We hypothesized that lethal amounts of endotoxin may disturb the disaggregation process in non-RE organs, thereby causing abnormal deposition of platelet-endotoxin complexes which could have locally damaging effects. Persistence of endotoxin-platelet aggregates in the vasculature of non-RE organs has previously been associated with toxicity (1, 15), and animals made tolerant to the lethal effects of endotoxin demonstrate improved RE clearance of the toxin (1, 10). Platelet responses to endotoxin may be an important factor in such improved RE clearance. In this paper we will provide evidence that platelets from tolerant rabbits have different aggregation characteristics from platelets of animals that are not resistant to the lethal effects of endotoxin. These new aggregation characteristics in tolerant animals may promote more efficient transport of endotoxin-platelet complexes to the RE system.

MATERIALS AND METHODS

Induction of tolerance. Adult New Zealand white rabbits (2.5 kg) were made tolerant to *Salmonella typhosa* endotoxin (lipopolysaccharide W, Difco) by receiving five daily intraperitoneal injections of the toxin (0.2, 0.3, 0.4, 0.5, and 0.5 mg). The effectiveness of this regimen was determined by intravenous challenge of the rabbits with a lethal dose of endotoxin (0.15 mg/kg). Animals that had been made tolerant survived this dose of toxin.

Blood collection and preparation. Platelet-rich plasma was prepared from blood obtained by cardiac puncture. Animals were fasted overnight and anesthetized with metofane (methoxyflurane; Pitman-Moore, Inc.) before puncture. Heparin (Upjohn) was used as an anticoagulant (10 U/ml). Plastic equipment was used in handling all blood and plasma samples.

Platelet-rich plasma was obtained by centrifugation of whole blood at 300 × *g* for 10 min at room temperature (22 to 26°C). Approximately 850,000 platelets per mm^3 were obtained in preparations from either tolerant or normal rabbits. A quantity of 1 to 2 ml of platelet-rich plasma supernatant fluid was left in each centrifuge tube to reduce the possibility of drawing erythrocytes into the platelet fraction collected. Platelet-free plasma was obtained by centrifuging that frac-

tion remaining after platelet-rich plasma was withdrawn at 1,200 × *g* for 10 min at room temperature.

Platelet aggregation. Aggregation of platelets was determined on 1-ml samples of platelet-rich plasma in plastic cuvettes equipped with plastic-covered magnetic stirring disks. Increased light transmission (aggregation) was recorded from a Beckman DU-2 spectrophotometer equipped with a magnetic stirrer and 37°C water bath.

A base-line recording (utilizing a strip chart recorder) of platelet-rich plasma was obtained for 1 to 2 min to allow the solution to warm to 37°C. Sensitivity to adenosine 5'-diphosphate (1 μg/ml)-induced aggregation was determined for each daily batch of platelets. Endotoxin was added to the cuvettes with a Hamilton 10-μl syringe. The time from injection of toxin into the cuvette until the onset of aggregation was determined and will be referred to as "lag time."

The pattern of the aggregation was also followed. If the pen never returned toward the base line, aggregation was termed "secondary" or "irreversible." Tracings in which the recorder pen returned toward the base line after reaching a maximum were termed "primary" or "reversible" aggregations.

RESULTS

Platelet aggregation characteristics in tolerant and nontolerant rabbits. Rabbit platelets were responsive to as little as 0.5 to 1.0 μg of *S. typhosa* endotoxin. Differences in platelet aggregation characteristics between tolerant and nontolerant rabbits were studied with 10 or 100 μg of endotoxin. At these concentrations of endotoxin, platelets from tolerant animals had a more rapid onset of aggregation than those from nontolerant animals (Table 1). Lag times for platelets from the tolerant group were approximately 90 s shorter than those from nontolerant rabbits.

When platelets from normal rabbits were maximally aggregated, no reversal of the aggregation process was observed with platelets obtained from tolerant rabbits. Platelet responses associated with tolerance were obtainable for periods of 1 to 3 weeks after the last injection of endotoxin.

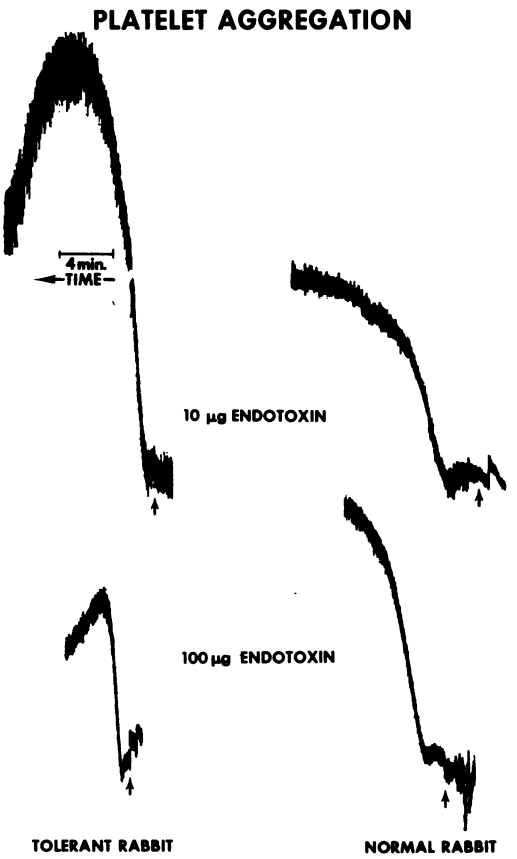

FIG. 1. *Aggregation characteristics of platelets from tolerant and nontolerant rabbits. Arrows indicate point at which endotoxin was injected into the cuvette. The time elapsed between the arrow and the onset of aggregation is approximately 40 and 130 s for tolerant and nontolerant preparations, respectively.*

TABLE 1. *Aggregation response time[a] for rabbit platelets exposed to endotoxin[b]*

Endotoxin (μg)	Prepn to which endotoxin was added[c]				
	N-PRP	T-PRP	N-PRP/N-PPP	T-PRP/-N-PPP	N-PRP/-T-PPP
10	146 ± 26 (7)	43 ± 4 (12)		38 ± 5 (5)	42 ± 10 (4)
100	121 ± 14 (9)	34 ± 4 (20)	184 ± 16 (3)	56 ± 8 (3)	43 ± 6 (4)

[a] Expressed as mean seconds ± standard error. Number in parentheses is the number of determinations from which the mean was derived.

[b] *S. typhosa* lipopolysaccharide W, Difco.

[c] N and T, Material from normal and tolerant rabbits, respectively. PRP, Platelet-rich plasma; PPP, platelet-poor (cell-free) plasma. The double designations (e.g., N-PRP/N-PPP) indicate that the two preparations were mixed 1:1 before addition of endotoxin.

Survival studies. Rabbits that had undergone endotoxin treatments to induce tolerance and had platelet aggregation characteristics associated with tolerance were challenged intravenously with a normally lethal dose of endotoxin (0.15 mg/kg). The survival of these animals was compared with that of rabbits that had not been previously treated but were similarly challenged with endotoxin. All nontolerant animals (five of five) died within 24 h of challenge, whereas only one out of six of the tolerant animals succumbed to the challenge with endotoxin.

Contribution of plasma factors to platelet responsiveness to endotoxin. Platelet-free plasma from tolerant rabbits was added (1:1) to platelet-rich plasma from nontolerant animals approximately 5 min before aggregation characteristics of the platelets were tested. The reverse mix was also made, and the results from both mixes were compared with those obtained for the original platelet preparations before dilution. Addition of plasma from tolerant rabbits to platelets from normal animals conferred tolerant characteristics on these platelets. After dilution with plasma from tolerant animals, the platelets had shorter lag times (Table 1) than previously and were able to reverse the aggregation process (Fig. 2). This effect was also obtained when *Escherichia coli* endotoxin was used to induce aggregation instead of *S. typhosa* toxin. When platelets from tolerant animals were similarly diluted with plasma from nontolerant rabbits, no changes in aggregation patterns were observed.

DISCUSSION

We found that platelets from rabbits that have been made tolerant to the lethal effects of endotoxin have aggregation characteristics different from those of platelets from nontolerant animals. Platelets from tolerant rabbits responded more rapidly to the aggregating effects of endotoxin than did platelets from nontolerant animals. Furthermore, unlike platelets from nontolerant rabbits, platelets from tolerant rabbits aggregated reversibly. These characteristics of platelets from tolerant rabbits may enhance RE clearance of endotoxin and thereby promote survival.

Since platelet-free plasma from tolerant rabbits could confer tolerant characteristics on platelets from nontolerant animals, a humoral factor must be principally responsible for altered platelet responsiveness to endotoxin. The nature of this factor is unknown. Inflammation in rabbits induces an increase in C-reactive protein which limits platelet aggregation (6, 13). Prostaglandins can also alter platelet aggregation (12, 14). Endothelial cells of non-RE tissues (17) and granulocytes (7) release fibrinolytic factors that could also influence platelet aggregation. Anti-endotoxin antibody has been associated with tolerance (8).

Enhanced uptake of endotoxin by the livers of tolerant animals may result from improved splanchnic blood flow and enhanced phagocytosis by hepatic cells (1). Based on our findings, this more efficient RE sequestration of endotoxin could also be due to more effective transport of endotoxin to the liver by platelets. In addition, platelet aggregates are known to release substances that enhance phagocytic function of cells of RE tissues (4).

The platelet aggregation characteristics induced by making the rabbits tolerant to endotoxin lasted 1 to 3 weeks after the final injection of endotoxin. A similarly variable period was observed by others for persistence of survival tolerance in rabbits (1). However, tolerance to the colony-stimulating factor response induced by endotoxin in mice lasts for more than a year (16). It is possible that distinct factors are re-

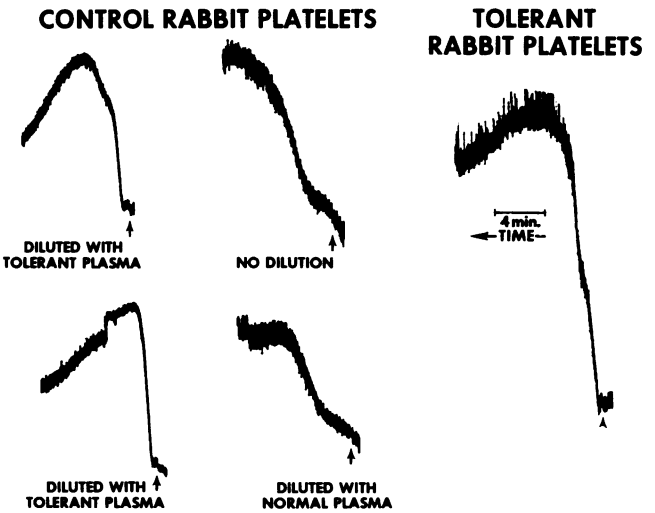

FIG. 2. *Alteration of aggregation characteristics of platelets from nontolerant rabbits by dilution with plasma from tolerant rabbits. Arrows indicate point at which endotoxin was injected into the cuvette. The time elapsed between the arrow and the onset of aggregation is approximately 40 and 130 s for tolerant and nontolerant preparations, respectively.*

sponsible for different types of tolerance (9).

The cause-and-effect sequence in the development of tolerance is difficult to determine. A humoral factor influencing the responsiveness of platelets to endotoxin could also affect other cells. For example, in rabbits that have been made tolerant to the lethal effects of endotoxin, leukocyte migration is no longer inhibited (2). This response could be due, however, to adaptation of platelets rather than leukocytes to endotoxin, since we have found that human platelets can inhibit chemotactic responsiveness of granulocytes (J. M. Sheil and R. I. Walker, manuscript in preparation). Finally, if accelerated transport of endotoxin to the RE system is induced by a factor affecting platelet aggregation, this effect may be even more beneficial if RE macrophage responsiveness to endotoxin has been altered by a factor released from platelets or other cells.

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