Augmentation by Platelets of Granulocyte Aggregation in Response to Chemotaxins: Studies Utilizing an Improved Cell Preparation Technique

By Heinz Redl, Dale E. Hammerschmidt, and Günther Schlag

Considerable evidence exists to suggest roles for both platelets and granulocytes (PMNs) in pulmonary injury in shock. While believing that the major contributions of the two cell types are sequential, in vitro observations suggested that direct interactions between granulocytes and platelets might also amplify tissue damage. Using isotonic Percoll density gradients to isolate PMNs, we therefore studied the effect of deliberate platelet contamination on PMN aggregation. PMN aggregation in response to N-formyl-met-leu-phe or activated complement was enhanced by the presence of 1 platelet/PMN, an effect that became maximal at 16 platelets/PMN (p < 0.01); large mixed aggregates were formed. Lysed, aspirinated, and indomethacin-treated platelets retained their augmentative capacity, as did platelets washed by gel filtration. The effect was not mimicked by the addition of histamine or serotonin to PMN preparations. None of these platelet preparations augmented lysosomal enzyme release. We conclude that platelets augment PMN aggregation, both by forming giant mixed PMN/platelet aggregates and also by producing a labile augmentative substance, the production of which may be independent of thromboxane synthesis. We propose that direct as well as sequential platelet/PMN interactions may be important in tissue injury in shock.

A CONSIDERABLE BODY of evidence suggests that activated granulocytes (PMNs) may play a role as mediators of tissue injury in such diverse states as lung dysfunction during extracorporeal circulations, myocardial infarction, and the complications of shock, such as "shock lung." In fact, using special biopsy techniques to study polytrauma patients within the first few hours after injury, the earliest changes we have found in the lungs have been extensive leukostasis, with PMN degranulation and endothelial cell swelling. A mechanism for such leukostasis is suggested by the fact that granulocytes aggregate when stimulated either in vitro or in vivo, and thus have the potential for embolization to the microvasculature; there they might cause transient mechanical circulatory compromise, while triggering structural damage to the microvessels through the release of proteases and toxic oxygen compounds.

Evidence also exists that platelets and the plasma coagulation cascade are important in the same syndromes. Thus, the formation of platelet aggregates at the site of critical coronary artery stenosis has been described in experimental animals, and has led to large-scale trials of antiplatelet drugs in atherosclerotic diseases. Further, platelets and fibrin are sometimes found in the lung in the late stages of shock-associated lung injury.

It has therefore long seemed likely in the above-mentioned syndromes that both platelets and PMNs are important; direct interactions between the two cell types have therefore also seemed possible. This possibility is further suggested by the detection of mixed masses of PMNs and platelets in the lungs of experimental animals subjected to shock or anaphylatoxin instillation and indirectly by the formation of mixed PMN/platelet aggregates in vitro in blood from patients with thrombotic states or with the fat embolism syndrome, as reported by Silbergleit. Other workers from the Hematology Section at the University of Minnesota have supported the concept of direct interaction in finding that the presence of platelets or platelet release products augmented injury of cultured endothelial cells wrought by stimulated granulocytes, this augmentation seemed to be due to an enhancement of neutrophil adherence to endothelial cells, an effect also reported to occur in a nylon-fiber assay of PMN adherence. Noting that some of the "granulocyte" aggregates formed in vivo were very large, and postulating that they might in fact be mixed platelet/PMN aggregates, we studied the effect of deliberate platelet contamination on the aggregation of PMNs in vitro.

MATERIALS AND METHODS

Granulocytes were separated from the heparinized (≤4 U/ml) venous blood of normal volunteers using a Percoll gradient in the following fashion: in a 50-ml polypropylene centrifuge tube, 15 ml of venous blood was centrifuged at 1000 g for 15 minutes. The platelet-depleted fraction was washed and resuspended in basal buffer, and 1 ml of the washed granulocyte fraction was added. This suspension was then overlaid with Percoll gradient and centrifuged at 1000 g for 10 minutes. The granulocytes were collected and resuspended in basal buffer. The granulocytes were then incubated with indicated stimuli and the aggregation was monitored using a Coulter counter. The aggregation was monitored in a Coulter counter. The aggregation was monitored in a Coulter counter.
of 55% Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) was layered over 15 ml of 74% Percoll; 15–20 ml of fresh, whole heparinized blood was then layered over the Percoll, and the tube was centrifuged at 400 g for 20 min at 20°C. The granulocytes appeared as a band of turbidity just above the infranatant erythrocytes and well separated from the supernatant mononuclear cells, platelets, and plasma. The granulocyte layer was carefully decanted, washed once in physiologic saline, and suspended (10³ PMN/ml) in calcium-and-magnesium-free Hanks' balanced salt solution (HBSS) containing 0.5% human serum albumin (HBSS/Alb). After the wash, a 20-sec hypotonic lysis of red cells was carried out, if needed. The resultant preparations reliably contained fewer than 2% platelets and mononuclear cells, and the harvested cells showed greater than 99% viability by Trypan blue exclusion. Cell yield ranged from 60% to 90%; viability persisted for a minimum of 8 h at room temperature (and was documented to exceed 95% at 18 h in one preparation).

Aggregation was observed and measured using a standard platelet aggregometer (Upchurch and Co, Leicester, England), at 900 rpm and 37°C. To 0.4 ml PMN suspension were added 0.05 ml cytochalasin B (50 μg/ml), 0.05 ml Ca⁺⁺/Mg⁺⁺ solution (7 mM CaCl₂ + 3.5 mM MgCl₂), and 0.10 ml platelet or control (vide infra). A 1:1 dilution of such a mixture with HBSS/Alb was used as the cell-poor calibration standard. Following a 3-min preincubation, the aggregating stimulus (vide infra) was added and the response recorded both as maximal wave amplitude within 5 min and as area under the 5-min aggregation wave (latter determined by planimetry (digital planimeter MOP-AMOZ, Kontron, Munich, Germany)).

Citrate platelet-rich (PRP) and platelet poor (PPP) plasmas were prepared by conventional techniques with PRPs adjusted in count to from 10,000 to 750,000 platelets/μl by the addition of PPP (this resulted in final platelet counts in the cuvettes of from 1500 to 115,000/μl when PPP was present). Platelet lysates were prepared by triply freezing and thawing PRP, and supernatant plasma was separated therefrom by centrifugation (10,000 g for 10 min). Platelets relatively free from plasma proteins were prepared by gel filtration of PRP over a 15-cm column of Sepharose 4B (Pharmacia), eluting with Ca⁺⁺- and Mg⁺⁺-free HBSS, and similarly preparing lysates and lysate supernatant fluids.

Zymosan-activated serum (ZAS) was prepared as previously described, without the addition of epinephrine-α caproic acid. N-formyl-methionyl-leucyl-phenylalanine (FMLP) (Peninsula, San Carlos, Calif.) was dissolved to 10⁻⁴ M in Krebs-Ringer’s solution, was stored frozen in 1-ml aliquots, and diluted when needed. The usual aggregating stimulus for these studies was 50 μl of full-strength ZAS or 50 μl of 10⁻⁴ M FMLP.

Sodium indomethacin was prepared by dissolving the drug (Sigma Fine Chemicals, St. Louis, Mo.) to 200 mM in 100 mM sodium carbonate, stirring for 20 min at 80°C, and restoring physiologic pH by the dropwise addition of 1 N HCl. For studies involving platelet aggregation, 1 volume of drug solution (dilutions prepared in physiologic saline) was added to 9 volumes of PRP and was incubated for 5 min at 37°C before the addition of the aggregating stimulus. For studies of granulocyte aggregation, 1 volume of drug solution was added to 9 volumes of the granulocyte/platelet or granulocyte/control mixture, and was incubated for 5 min at 37°C before the addition of the other reagents and the aggregating stimulus. In each case, the reported drug concentration is that achieved in the incubation with the cells.

50–100-μl aliquots of aggregated cell suspensions were placed on self-staining glass microscope slides (Boehringer-Mannheim, Vienna) and were examined and photographed by conventional light microscopy. Aliquots were also fixed in buffered glutaraldehyde, allowed to sediment at 1 g, and were prepared for electron microscopy as previously described. Myeloperoxidase was measured fluorimetrically in supernatant fluids from aggregation cuvettes, taken 4 min after the addition of the aggregation stimulus; values were expressed as a percentage of the total peroxidase content of a lysate of a like volume of the same cell preparation. Lysozyme was measured and similarly compared with the total enzyme content of a lysate.

RESULTS

Granulocytes prepared in the above manner, to which 0.1 ml PPP had been added, aggregated upon addition of 50 μl ZAS or 50 μl 10⁻⁴ M FMLP, as has been previously reported. However, the waves were of somewhat lower amplitude, and the oscillation amplitude was generally less than are seen in studies using cells harvested by Ficoll-Hypaque centrifugation following hypotonic lysis of erythrocytes. When PRP from any of 10 normal donors was substituted for PPP, an increase in wave amplitude (and often in oscillation amplitude) was observed (Fig. 1). The wave amplitude at 5 min was approximately doubled (range 1.3–3.6-fold augmentation of amplitude) when 8–16 platelet-free PRP dilution artefact.

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Fig. 1. The presence of platelets augments the aggregation response to granulocytes. As in platelet aggregometry, the vertical axis is an arbitrary scale of light transmission; polarity is such that downward deflections indicate increased transmission (T), implying aggregation. The horizontal axis depicts time. The addition of FMLP produces an aggregation wave in PMNs devoid of platelets (uppermost wave). The addition of as few as 2 platelets/PMN augmented this response in cells from most normal donors (lower most wave). The effect was maximal by 8–16 platelets/PMN (lower two tracings), approaching the ratio that obtains in whole blood. Not displayed, a similar family of waves was generated when zymosan-activated serum was used as the aggregating stimulus. The addition of FMLP to PRP, or to a diluted PRP equal in platelet count to the 8:1 or 16:1 preparations, produced only a dilution artefact.

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Fig. 2. Gross, light microscopic, and electron microscopic demonstration of enhanced PMN aggregation in the presence of platelets. (Left) After the addition of FMLP or zymosan-activated serum, PMN preparations containing 4 platelets/PMN formed frankly macroscopic aggregates (left cuvette); in contrast, those free from platelets showed only a somewhat more granular appearance (right cuvette) than unstimulated preparations. (Center) By light microscopy, individual aggregates formed in the presence of platelets were found to contain far more PMNs than those formed in the absence of platelets. This aggregate, formed with 4 platelets/PMN, contained in excess of 200 PMNs; platelet-free aggregates seldom contained more than 30 cells. Reference line = 50 μm. (Right) From the absence of platelets in supernatant fluids of mixed aggregation experiments, we inferred that the aggregates were mixed. This was confirmed by electron micrographs such as this, showing both cell types in the aggregate. Reference line = 10 μm.
lets were present per PMN, as was the area under a 5-min aggregation wave. A stepwise diminution of effect was noted as the number of platelets was decreased (Fig. 1) (p for maximal effect 0.001, Mann-Whitney U-test). The addition of similar amounts of zymosan-activated plasma or FMLP to platelet-rich plasma or gel-filtered platelets in the absence of granulocytes did not produce discernible aggregation waves.

When such aggregates were examined grossly, it was found that the platelet-containing preparations in which the platelet/PMN ratio exceeded 4 had frankly macroscopic aggregates (Fig. 2, left panel); those lacking platelets had only a somewhat more granular appearance than unstimulated preparations. Correspondingly, light microscopic examination showed aggregates formed in the absence of platelets seldom to contain more than 15 cells (not shown); those from the platelet-containing incubations were in comparison huge, often containing in excess of 100 cells (Fig. 2, center panel). Few platelets were noted in the suspending medium in such preparations; the resulting suspicion that the aggregates were mixed was confirmed by transmission electron microscopy (Fig. 2, right panel).

Enhancement of aggregation was seen both in the presence and absence of cytochalasin-B. In its presence, however, we measured release of myeloperoxidase and lysozyme and found it to be no different when platelets were present. That is, in the presence or absence of added platelets, the addition of FMLP to PMNs led to 34.5% (±5.9% SEM) release of lysozyme and a 30.0% (±6.0%) release of myeloperoxidase.

The means by which platelets worked their augmentative effect was further explored. Gel-filtered platelets suspended in HBSS/Alb promoted PMN aggregation to the same extend as did a similar number of platelets provided as PRP. Lysates of platelets and supernatant fluids from lysates of platelets (prepared either in plasma or in HBSS/Alb) augmented PMN aggregation as well (Fig. 3). Platelets from a donor who had taken aspirin (0.5 g p.o. q.d. for 2 days) also augmented PMN aggregation, suggesting that thromboxane synthesis was not of central import. Supporting this suggestion, the incubation in PRP of 140 μM sodium indomethacin led to the complete abolition of the secondary wave of platelet aggregation on the subsequent addition of 1/9 volume of 70 μM ADP; in contrast, 14 mM Na indomethacin was required to reduce the PMN aggregation wave amplitude in the presence of PRP to that obtained with PPP. Furthermore, the addition of as much as 10 μM serotonin to the incubation lacking platelets enhanced PMN aggregation only trivially (not shown; p ~ NS). Supernatant plasma from PRP in which platelet aggregation had been provoked by 16 μM ADP or 1 μg/ml collagen (concentrations achieved in the cuvette) did not aggregate PMNs, nor did it augment PMN aggregation on the subsequent addition of zymosan-activated serum or FMLP. Thus, the mediator of augmented aggregation seems not to be released during platelet aggregation in any quantity adequate to influence PMN aggregation without direct cell-to-cell contact.

Aggregates formed when supernatant fluids from high-speed centrifugation of platelet lysates were added to PMNs were also unusually large, mimicking grossly those seen in the presence of platelets (Fig. 2). Obviously, these could not be mixed aggregates, since platelets were not present. Electron micrographs were therefore prepared of such aggregates and were scored by an observer unaware of their method of preparation. Aggregates generated in the presence of platelet lysates (or supernatant fluids therefrom) (Fig. 4, left panel) were much denser than those prepared in the absence of platelets or platelet products (Fig. 4, right panel). That is, the number of nuclei per unit area and the proportion of cytoplasmic membrane per cell in contact with an adjacent cell were each significantly
The presence of platelets leads to denser, as well as larger, PMN aggregates. Reference lines each = 10 μm. (Left) Aggregates formed in the presence of platelet lysates or supernatant fluids therefrom are quite dense. There are many nuclei per unit area of a micrograph, and the proportion of cell membrane that is in contact with adjacent cells is high. (Right) In contrast, aggregates formed in the absence of platelets are comparatively loose, having fewer nuclei per unit area and having a low proportion of cell membrane in contact with adjacent cells. That this difference is real was confirmed by morphometry performed by an observer unaware of the method of preparation of each micrograph.

greater in the presence of platelet products than in their absence (semiquantitative scoring, p < 0.05 by Mann-Whitney U-test). Aggregates formed in the presence of whole platelets (Fig. 2, right panel), although they could not be scored "blindly" because of the obvious presence of platelets, showed intermediate density.

The identity of the messenger substance from the platelet remains to be determined. However, it appears to be soluble (not removed by high-speed centrifugation) and proved labile on heating (60°C for 1 hr) or prolonged (48 hr) storage at room temperature (in plasma or HBSS at pH = 7.4).

DISCUSSION

From a variety of clinical and pathologic parallels (summarized elsewhere) we have postulated an important role for the stimulated granulocyte in a variety of clinical states, including posttraumatic lung dysfunction. However, there also exists an extensive body of literature suggesting that platelets and the plasma coagulation system are important as well. We have previously reported (both in traumatized humans and in experimental animals) that leukostasis and endothelial injury precede interstitial edema and the deposition of fibrin in shock. Thus, our original...
view of the PMN–platelet interaction in shock was one of sequential contributions, the PMN being an early effector of endothelial injury, and platelets and coagulation factors being important somewhat later. However, it is also quite possible that platelets and granulocytes might interact directly with one another in tissue injury in shock, a postulate supported by the observation of Boogaerts et al. that PMN-mediated endothelial cell damage is augmented—seemingly via enhanced adhesion—in the presence of platelets or platelet release products.

Several previous attempts have failed to demonstrate enhancement of PMN aggregation in the presence of platelets (unpublished data, D.E.H. et amici) using the originally described modification of the cell preparation technique of Bøyum. In that method, over 2 hr are consumed in cell preparation, so that the cells are already relatively “old” by the time an experiment actually begins, and the cells experience a brief period of extreme hypotonicity, followed by a long period of hypertonicity. Furthermore, while the modified Boyum method is capable of producing cell preparations with fewer than 2% contaminating platelets, in practice, the contamination is often far more extensive. In contrast, the current technique routinely results in cell preparations with fewer than 2% platelets, takes less than an hour, and subjects the granulocyte to no osmotic stress (unless red cell contamination is excessive, in which case osmotic stress is limited to 20-sec hypotonicity).

Using this improved cell preparation technique, we found it easy to demonstrate augmentation of PMN aggregation in the presence of added platelets. Thus, the addition of as few as 1 platelet per PMN led to discernible enhancement of PMN aggregation wave amplitude, an effect that became maximal as the platelet/PMN ratio of whole blood was approached (Fig. 1). It should be noted that the mere presence of inert particles would create optical interference that would tend to minimize rather than magnify wave amplitude (a phenomenon all too familiar to those of us who occasionally have prepared cells with excessive erythrocyte contamination!). That this effect was also not simply the simultaneous and separate aggregation of the added platelets was also demonstrated: the addition of ADP (final concentration in the cuvette 1–8 μM) or collagen (1 μg/ml) to mixed preparations produced perceptible aggregation waves only when the number of platelets was severalfold that required to enhance PMN aggregation (not shown); such waves were equal to or smaller than the waves obtained with a like number of platelets alone.

Furthermore, the fact that aggregation was enhanced was readily apparent upon gross, light microscopic and electron microscopic examination of aggregates. Those formed in the presence of large numbers of platelets were far larger and had great numbers of platelets incorporated into them (Fig. 2).

It was not necessary for platelets to be intact for them to work their effect; indeed, platelet lysates and the supernatant fluids therefrom proved equally efficacious with the number of whole platelets from which they were prepared. From earlier observations in cultured endothelial cell injury and PMN adherence to nylon fibers, we had expected the augmentation to depend on thromboxane synthesis and serotonin release. Such was not the case, however; both aspirin-treated and indomethacin-treated platelets, the latter demonstrated to have secondary aggregation blocked, functioned as well as normals in augmenting granulocyte aggregation and added serotonin did not mimic the effect. The possibility that lipooxygenase—rather than cyclooxygenase—pathway products are important in PMN/platelet interactions is attractive and has certainly not been excluded by these studies.

We conclude that platelets augment granulocyte aggregation through the formation of mixed aggregates far larger than the aggregates observed in absolutely platelet-free preparations. The ability of supernatant fluids from platelet lysates to also augment aggregation of PMNs suggests that there might be a substance released from platelets—other than serotonin or thromboxane A2—which works the effect. We postulate that such platelet/granulocyte interactions may not be limited to aggregation and might be of central importance in the genesis of certain complications of shock; further, they may well explain some of the seemingly discrepant observations favoring a role for one cell type or another in experimental shock models.

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REFERENCES

Augmentation by platelets of granulocyte aggregation in response to chemotaxins: studies utilizing an improved cell preparation technique

H Redl, DE Hammerschmidt and G Schlag