Interaction with Autologous Platelets Multiplies Interleukin-1 and Tumor Necrosis Factor Production in Mononuclear Cells

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The effect of activated platelets on cytokine production by human peripheral blood mononuclear cells (PBMC) was investigated. When PBMC were cocultivated with activated autologous platelets amid lipopolysaccharide (LPS, 50–100 pg/mL) for 8 h, the production of interleukin (IL)-1α increased 11- to 18-fold and tumor necrosis factor (TNF)-α 3- to 5-fold compared with PBMC without platelets. Activated platelets in a dual-chamber well that prevented platelet-PBMC contact but permitted passage of soluble factors enhanced IL-1α production (P < .01). Platelet-PBMC contact in the chamber resulted in a further enhancement of IL-1α production. These data suggest that platelet-PBMC interaction, both directly and with platelet-derived factors, enhances production of shock-producing IL-1α and TNF-α, albeit differently. The interaction of platelets with monocytes may play an important role in the pathophysiology of sepsis and disseminated intravascular coagulation.

Gram-positive and -negative septicemias remain serious clinical problems that are often complicated by disseminated intravascular coagulation (DIC) [1–3]. Interleukin (IL)-1 and tumor necrosis factor (TNF)-α are key mediators in the pathogenesis and lethality of both types of sepsis [4, 5]. In experimental animals, both IL-1 and TNF in the circulation are elevated after injection with either gram-positive or -negative organisms and are considered to be a common pathway resulting in hemodynamic decompensation and mortality [6]. Similar findings have been reported in humans with sepsis, and furthermore, plasma levels of both IL-1β and TNF-α can be significantly higher in patients with DIC than without DIC [7, 8].

We reported that IL-1 receptor antagonist (IL-1Ra) improved hemodynamic shock in rabbits injected with either Escherichia coli or Staphylococcus epidermidis [9, 10]. We found that the circulating levels of TNF-α in rabbits treated with IL-1Ra in S. epidermidis-induced shock decreased compared with levels in saline-treated animals, suggesting that TNF-α production may be dependent on IL-1 induced in an early phase of gram-positive sepsis. In the same model, a dramatic drop in the circulating numbers of platelets was observed in both treatment groups after infusion of S. epidermidis. Thrombocytopenia and neutropenia are frequently observed in septicemia. Hawrylucowicz and colleagues [11, 12] reported that activated platelets express cell-associated IL-1–like activity that is capable of inducing intercellular adhesion molecule 1, IL-6, and granulocyte-macrophage colony-stimulating factor produced by endothelial cells. Another report indicated that activated platelets induce IL-8 secretion by endothelial cells via membrane-associated IL-1 activity [12a]. Moreover, activated platelets can interact with monocytes and neutrophils through P-selectin (GMP-140, PADGEM, CD62). P-selectin is stored in granules of resting platelets as an integral membrane protein and is translocated to the plasma membrane after platelet activation [13–15].

Recently, Heddle et al. [16] reported that cytokines generated by leukocytes may be responsible for febrile, nonhemolytic transfusion reactions. These reactions occur in 30% of platelet transfusions and are positively correlated with increased leukocyte contamination [17]. They are observed in 1% of red cell transfusions, even though these have much higher leukocyte concentrations [17]. If platelet-monocyte interactions enhance cytokine production, then this interaction may be involved in the febrile reaction to transfused platelets.

In this study, we investigated in vitro the interaction of platelets with peripheral blood mononuclear cells (PBMC) and their effect on cytokine production in the presence of low concentrations of lipopolysaccharide (LPS), as seen in the circulation of patients with gram-negative sepsis. We also investigated the role of platelets on the synthesis of cytokines by PBMC stimulated with gram-positive S. epidermidis and determined if platelets interacting with monocytes play an important role in shock seen in sepsis and DIC.

Materials and Methods

Materials. Ficoll type 400, TRIS-HCl, trisodium citrate, and LPS (E. coli O127:B8) were obtained from Sigma (St. Louis). Hypaque-M (90%) was obtained from Winthrop Breon Labora-
Aliquots of the bacterial suspension were stored at -70°C until used in experiments.

Preparation of polymyxin B on TNF-α production by PBMC stimulated with S. epidermidis was prepared as described [6]. S. epidermidis was washed three times with pyrogen-free saline and the organisms were inoculated into brain-heart infusion broth and propagated at 37°C over night with shaking. After centrifugation, the S. epidermidis pellet was washed three times with pyrogen-free saline and the organisms were counted; then bacteria were killed by boiling for 30 min. Bacteria were washed six more times with sterile saline and resuspended at 10^6/mL in ultrafiltered RPMI 1640 as above. The concentration of bacteria was determined spectrophotometrically and confirmed by direct counting in a Petroff-Hausser bacteria counter. Aliquots of the bacterial suspension were stored at -70°C until used in experiments.

To confirm whether the suspension of S. epidermidis in RPMI 1640 was contaminated with endotoxin, we investigated the effect of polymyxin B on TNF-α production by PBMC stimulated with S. epidermidis (200 bacteria/PBMC). LPS (1 ng/mL) was used as a positive control. The S. epidermidis suspension and LPS solution were separately preincubated with or without polymyxin B at 5 μg/mL for 1 h at room temperature. Then PBMC (1.2 × 10^6 cells/mL, final concentration) were added to these samples and incubated for 8 h. Polymyxin B did not affect TNF-α production by PBMC stimulated with the S. epidermidis, while polymyxin B completely inhibited LPS-induced TNF-α synthesis.

Results

Effect of activated platelets on IL-1α and TNF-α production by PBMC in the absence of LPS. To investigate whether thrombin-activated platelets alone induce IL-1α or TNF-α by PBMC, PBMC were incubated without LPS in the presence of thrombin (0.15 U/mL, final concentration), thrombin-activated platelets, or nonactivated platelets (5 × 10^7 cells/mL, final concentration) for 4, 8, 16, and 24 h (data not shown). In the absence of LPS, there was no induction of PBMC IL-1α or TNF-α production by thrombin alone or by platelets with or without thrombin. After 16 and 24 h of incubation, there was, however, a small but statistically significant (P < .01) reduction of TNF-α in the presence of thrombin-activated platelets compared with PBMC stimulated by thrombin alone (platelets omitted from incubation).

Effect of activated platelets on LPS-induced synthesis of IL-1α and TNF-α. As shown in figure 1, when PBMC were incubated with platelets activated during isolation or further activated with thrombin (0.15 U/mL) in the absence of LPS for 8 h at 37°C, IL-1α and TNF-α were barely detected. In the
absence of platelets, PBMC incubated with a low concentration of LPS for 8 h at 37°C produced small amounts of IL-1α and TNF-α. When PBMC and thrombin-activated platelets were coincubated in the presence of LPS for 8 h at 37°C, an 11- to 18-fold increase in IL-1α and a 3- to 5-fold increase in TNF-α were observed compared with results from PBMC incubated with LPS alone.

To ascertain whether 0.15 U/mL thrombin induces IL-1α or TNF-α, PBMC were incubated with thrombin in the presence or absence of LPS for 8 h at 37°C. Thrombin (0.15 U/mL) did not affect either IL-1α or TNF-α production (figure 1). These results demonstrate that thrombin-activated platelets, but not thrombin alone, augment the production of IL-1α and TNF-α by PBMC stimulated by LPS at low concentrations. Platelets not treated with thrombin, activated 25%-30% for P-selectin expression, enhanced IL-1α production 6- to 14-fold and TNF-α production 3-fold when coincubated with PBMC and LPS for 8 h.

Effect of platelets on S. epidermidis–induced synthesis of IL-1α and TNF-α. Thrombin-activated platelets (5 × 10⁸/mL) alone did not induce IL-1α or TNF-α production by PBMC incubated in the absence of S. epidermidis, even after 24 h of incubation (data not shown). To investigate whether platelets augment IL-1α and TNF-α production by PBMC in the presence of S. epidermidis, PBMC and platelets were coincubated with various concentrations of S. epidermidis (2 or 200 bacteria/PBMC) for 8 h at 37°C. As shown in figure 2, PBMC (1.2 × 10⁶/mL) incubated with platelets (5 × 10⁸/mL) in the presence of 2 S. epidermidis bacteria/PBMC for 8 h induced only minimal IL-1α and TNF-α synthesis.

However, when PBMC and platelets (either strongly activated with thrombin or non–thrombin-treated but activated during isolation) were coincubated in the presence of 200 bacteria/PBMC for 8 h at 37°C, IL-1α production was enhanced 4-fold with thrombin-activated platelets and 2.7-fold with non–thrombin-activated platelets (from 1610 ± 342 pg/mL to 6514 ± 1058 and 4360 ± 533 pg/mL, respectively; P < .05) compared with IL-1α production by PBMC incubated with S. epidermidis without platelets. TNF-α production was enhanced 2.7-fold with thrombin-treated platelets and 2.2-fold with non–thrombin-activated platelets (from 418 ± 80 pg/mL to 941 ± 201 and 1121 ± 241 pg/mL, respectively; P < .05) compared with the production of TNF-α by PBMC incubated with S. epidermidis without platelets.

Effect of platelet concentration on LPS-induced cytokine production by PBMC. We next investigated the effect of the concentration of activated platelets on cytokine production by PBMC stimulated with LPS. When PBMC were incubated with 100 pg/mL LPS, the synthesis of IL-1α in PBMC was increased by activated platelets in a concentration-dependent manner (figure 3A). However, in contrast to IL-1α, TNF-α production was not enhanced by 10⁶ platelets/mL, although a significant increase in TNF-α production was observed with the addition of 10⁸ platelets/mL (figure 3B). When PBMC were replaced with MonoMac6 monocyteic cells, results similar to those in figure 3 were observed (not shown). Platelets at 10⁷/mL and 10⁸/mL enhanced TNF-α, whereas platelets at 10⁹/mL resulted in decreased TNF-α production.

Effect of platelet concentration on S. epidermidis–induced TNF-α production by PBMC. We next investigated the effect of differing numbers of platelets on S. epidermidis (200 bacteria/PBMC)–induced TNF-α synthesis by PBMC. S. epidermidis–induced TNF-α production was augmented by the presence of platelets in a platelet-number–dependent manner (figure 4). Platelets at 10⁷/mL as well as 5 × 10⁸/mL caused 3.0-fold increases in TNF-α production (P < .05). Platelets at
Role of soluble products of platelets and direct platelet-PBMC interaction in enhancing IL-1α and TNF-α production.

To determine whether platelet-derived soluble factors augment the production of cytokines, or whether the direct interaction of platelets with PBMC is needed, PBMC were incubated with but kept separate from platelets by a membrane filter in a dual-chamber culture well. This membrane filter prohibits direct PBMC-platelet contact but allows soluble factors to diffuse between the two chambers. When PBMC and activated platelets were placed in separate chambers, the production of IL-1α was significantly increased compared with PBMC with LPS but without platelets (figure 5A). Furthermore, incubation of PBMC with thrombin-activated platelets in the same chamber resulted in greater enhancement of IL-1α production (P < .01). In contrast, thrombin-activated platelets separated by a membrane enhanced the production of TNF-α as much as did incubation of PBMC in direct contact with platelets, although these increases did not reach statistical significance compared with the cells cultured without platelets (figure 5B).

Discussion

In these experiments, the presence of activated platelets greatly enhanced IL-1α and TNF-α production by PBMC stim-
may be possible that concentrations of these cytokines were
with the detection of membrane-associated antigens, or that
were undetectable in platelet lysates by our RIAs. The limits
so low that RIA could not detect them, that platelets interfered
of cell-associated IL-1 bioactivity expressed by platelets, it
of detection for these are

press cell-associated IL-1 bioactivity within minutes of activa­
platelet interaction.

Figure 4. Platelets enhance TNF-α production in number-depen­
dent manner. Peripheral blood mononuclear cells (PBMC, 1.2 × 10⁶/
ml) were incubated with or without different nos. of platelets in
absence or presence of Staphylococcus epidermidis at 200 bacteria/
ml for 8 h (n = 5). Data are mean ± SE. Significant differences
between TNF-α production by PBMC stimulated with S. epidermidis
with or without platelets: * P < .05, by paired t test.

ulated with low concentrations of LPS (50–100 pg/mL) and by
S. epidermidis. These results suggest that increases in cytokine
production in gram-negative or -positive sepsis might be partly
attributable to activated platelets.

In preliminary experiments, platelets incubated for 8 h with­
out thrombin still enhanced LPS-induced PBMC production of
IL-1α and TNF-α. Over time, isolated platelets are spontane­
ously activated in RPMI culture medium, which contains cal­
cium. It is well known that calcium plays a central role during
platelet activation [22, 23]. Recent studies also show that the
interaction of endotoxin with platelets results in changes in the
platelet membrane and potentiates the activation of platelets
[24, 25]. Furthermore, LPS induces platelet-derived soluble
factors that augment the priming effect of LPS on the oxidative
response of human polymorphonuclear cells [26]. Staphylo­
cocci also activate platelets. Among bacteria, staphylococci are
one of the most potent stimuli for platelet activation, and heat­
killed staphylococci can activate platelets as much as living
organisms [27].

Our data suggest that cytokine production is enhanced by
soluble factors released from platelets and by direct PBMC­
platelet interaction. It was recently reported that platelets ex­
press cell-associated IL-1 bioactivity within minutes of activa­
tion [11, 12]. However, when we incubated 10⁶ platelets with
thrombin and LPS for 8 h at 37°C, then subjected them to three
cycles of freeze-thawing, IL-1α, IL-1β, and TNF-α antigen
were undetectable in platelet lysates by our RIAs. The limits
definition for these are ~40 pg/mL. In light of other reports
of cell-associated IL-1 bioactivity expressed by platelets, it
may be possible that concentrations of these cytokines were
so low that RIA could not detect them, that platelets interfered
with the detection of membrane-associated antigens, or that
platelets contain a precursor IL-1 that requires a convertase
from monocytes to become a detectable form.

The effects of platelet-PBMC interactions on LPS-induced
TNF-α production were not as dramatic as on IL-1α produc­
tion. The production of TNF-α was enhanced, but not signifi­
cantly, when PBMC were incubated with activated platelets in
dual-chamber culture wells (figure 5B). In addition, incubation
of PBMC with activated platelets at a concentration of 10⁹
cells/mL (yielding a higher ratio of platelets to PBMC than is
seen in peripheral blood) did not enhance TNF-α production,
although production of IL-1α was enhanced with high concen­
trations of platelets (figure 3A). At least two possibilities can
be considered for the variable effects of platelets on TNF-α
production. Transforming growth factor (TGF)-β, one of the
major cytokines released from platelets, induces neither IL-1
nor TNF-α production by PBMC alone. However, such low
concentrations of TGF-β as 0.1 ng/mL enhanced the production
of TNF-α by phytohemagglutinin- or LPS-stimulated PBMC,

Figure 5. Involvement of soluble mediators released from platelets
and platelet-PBMC (peripheral blood mononuclear cell) direct interac­
tions in enhancement of IL-1α and TNF-α production. PBMC (1.2
× 10⁶ cells/mL) stimulated by 100 pg/mL lipopolysaccharide (LPS)
were incubated with thrombin-activated platelets (2 × 10⁶ cells/mL)
kept separate (S) by membrane or in contact with each other (C) in
dual-chamber well (n = 6). A, Both soluble mediators produced by
platelets and platelet-PBMC direct contact are involved in enhance­
ment of IL-1α production seen with addition of platelets. B, TNF-α
production in both conditions was not significantly enhanced by plate­
lets. Data are mean ± SE. Significant differences: ** P < .01, by
paired t test.
while 10 ng/mL TGF-β inhibited the production of TNF-α [28, 29]. The effects of TGF-β, stimulatory or inhibitory, depend on the target cell type and the presence of other growth factors [28, 30]. It is possible that low concentrations of TGF-β may promote the production of TNF-α, while higher concentrations of TGF-β secreted from a larger number of platelets may abolish the effect of some other stimulatory factors produced by platelets.

Another possibility is that the release of proteolytic enzymes by activated platelets may degrade TNF-α in culture medium. Human platelets have several exopeptidases, such as aminopeptidase P, dipeptidyl peptidase IV, carboxypeptidase N, and angiotensin-converting enzyme [31]. It was recently reported that a dipeptidyl peptidase IV-like enzyme on the surface of human monocytic cells participates in TNF-α degradation [32]. Since the majority of monocyte IL-1α is intracellular, it would not be accessible to digestion by peptidases released from activated platelets. On the other hand, TNF-α would be degraded because the majority of TNF-α produced by PBMC is secreted. Thus, platelets may regulate cytokine levels not only by enhancing cytokine production but also by accelerating their degradation. Circulating cytokine levels, driven either by endotoxemia or other stimuli, may therefore represent the net of both positive and negative regulatory effects of platelets on monocyte cytokine production. Activated platelets release physiologically important substances in addition to TGF-β, such as platelet-derived growth factor, platelet-activating factor (PAF), and fibroblast growth factor. It remains to be determined whether other platelet products are associated with these effects on cytokine production. We are currently investigating the identities of the platelet-associated and supernatant modulating factors.

It is critical to note that this dramatic effect of platelets on the production of cytokines is seen in the presence of the low concentrations of LPS seen in the circulation clinically. The concentration of LPS in the circulation of patients with sepsis is generally <100 pg/mL. Low concentrations of gram-positive staphylococci were also used. Thrombocytopenia is frequently seen in cases of bacterial sepsis in humans and experimental animals. As soon as the vascular endothelium is injured, platelets adhere to the exposed subendothelial surface via glycoprotein Ib receptors [33], followed by generation of platelet plugs and blood clotting. Platelets are also activated by physiologic stimuli, such as adenosine diphosphate, epinephrine, collagen, or thrombin. Platelets express surface P-selectin minutes after activation, causing adherence with neutrophils and monocytes. Recently, Weyrich et al. [34] reported that activated platelets signal IL-8 and monocyte chemotactic protein-1 (MCP-1) production after coincubation with monocytes for 18 h [34]. This signaling is linked to RANTES, PAF, and P-selectin expression on the activated platelets. In this fashion, high concentrations of proinflammatory cytokines (IL-1α and TNF-α) and chemotactic factors (IL-8 and MCP-1) could be produced locally at the site of inflammation in the presence of gram-negative or -positive bacteria, contributing to tissue damage and organ failure.

Hemostasis is the major physiologic function of platelets. However, if the platelet and cytokine-producing mononuclear cell/macrophage interaction described here were selectively inhibited without destroying the ability to produce hemostasis, it might be of benefit in the prevention of organ failure and shock in sepsis and DIC.

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References


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