Human Neutrophil Degranulation During Extracorporeal Circulation


Cardiopulmonary bypass, especially when prolonged, may result in hemostatic failure and pulmonary dysfunction, which has been attributed to changes in platelets and leukocytes, respectively. It has been well documented that contact of blood with synthetic surfaces causes platelet activation. In this report, we explore mechanisms of the activation of neutrophils during simulated in vitro extracorporeal circulation and document the release of neutrophil lactoferrin and elastase during clinical cardiopulmonary bypass (CCB). Inhibition in the simulated circuit by prostaglandin E\(_2\) (PGE\(_2\)) and lidocaine suggests different mechanisms for release of neutrophil-specific proteins. During CCB with a bubble oxygenator it was observed that platelet counts fell to 42% ± 2% of baseline. In addition, \(\beta\)-thromboglobulin antigen (\(\beta\)-TG), a platelet-specific, \(\alpha\)-granule protein marker reflecting the release reaction, increased from 0.15 ± 0.05 to 0.84 ± 0.11 \(\mu\)g/mL. Neutrophil counts decreased to 67% ± 7% of prebypass levels but then gradually rose as bypass continued. Both lactoferrin, a neutrophil-specific granule marker, and neutrophil elastase, an azurophilic granule marker, increased in plasma threefold to 1.66 ± 0.33 \(\mu\)g/mL and 1.65 ± 0.68 \(\mu\)g/mL, respectively, just before bypass was stopped. When fresh heparinized human blood was recirculated within an extracorporeal membrane oxygenator bypass circuit for 120 minutes, plasma \(\beta\)-TG rose to 5.13 \(\mu\)g/mL, lactoferrin increased from 0.13 ± 0.04 to 1.62 ± 0.22 \(\mu\)g/mL, and neutrophil elastase rose from 0.05 ± 0.02 to 1.86 ± 0.41 \(\mu\)g/mL. At 120 minutes, lidocaine (100 \(\mu\)mol/L), which inhibits neutrophil activation, delayed release of lactoferrin (1.33 ± 0.26 \(\mu\)g/mL) and markedly inhibited release of elastase (0.24 ± 0.05 \(\mu\)g/mL) but did not inhibit release of \(\beta\)-TG antigen (5.66 \(\mu\)g/mL at 120 minutes). PGE\(_2\) (0.3 \(\mu\)mol/L) inhibited significantly the release of \(\beta\)-TG (0.31 \(\mu\)g/mL) and elastase (0.52 ± 0.11 \(\mu\)g/mL) and attenuated the release of lactoferrin (1.57 ± 0.45 \(\mu\)g/mL).

**MATERIALS AND METHODS**

CCB perfusion circuits contained a centrifugal pump (Biomedicus, Eden Prairie, MN), bubble oxygenator (BOS-102; Bartec Labs, Irvine, CA), and arterial line filter (model 1331) and cardiomyocyte reservoirs (Extracorporeal, Valley Forge, PA). SECC perfusion circuits (surface area of 0.9 \(m^2\)) including a spiral coil membrane oxygenator model 0800-2A (surface area of 0.8 \(m^2\)) and a venous reservoir (model R–500–4), were obtained from Sci-Med Life Systems, Minneapolis, and silicone tubing was obtained from Dow Chemical, Midland, MI. Beef lung heparin was obtained from Upjohn, Kalamazoo, MI; PGE\(_2\) and \(N\)-formyl-L-Methionine-L-Leucyl-L-Phenylalanine (FMLP) from Sigma Chemical, St Louis; and lidocaine from Elkins-Sinn, Cherry Hill, NJ. Flat-bottomed 96 well polystyrene microtiter plates (Immulon no. 2) were obtained from Dynatech Laboratories, Alexandria, VA. Goat antirabbit IgG (heavy and light chains) covalently coupled to biotin and peroxidase-conjugated avidin as well as peroxidase-conjugated goat antirabbit IgG (heavy and light chains) were obtained from Cappel Laboratories, West Chester, PA.

**Purified blood proteins.** \(\beta\)-Thromboglobulin antigen (\(\beta\)-TG) was purified to homogeneity as described.\(^5\) Purified human lactoferrin and monospecific rabbit antihuman lactoferrin were obtained from Calbiochem-Behring, La Jolla, CA. HNE was purified to homogeneity as described.\(^6\) Antibody raised in rabbits to HNE produced one precipitin arc either by immunodiffusion or immunoelectrophoresis against either purified HNE or a crude neutrophil extract. The HNE immunoassay utilized a 35% ammonium sulfate-enriched IgG fraction that was exhaustively dialyzed.

**Clinical cardiopulmonary bypass.** In ten patients from whom written informed consent was obtained, lactoferrin and HNE levels...
were measured in plasma samples obtained before, during, and after open-heart surgery for valvular or ischemic heart disease. In four of these patients plasma levels of platelet βTG were also sampled at the same time intervals. Blood samples (15 mL) were drawn from a radial artery catheter: (a) before induction of anesthesia; (b) after anesthesia, before heparin; (c) after heparin, before CCB; (d) five minutes after start of CCB; (e) 45 minutes after start of CCB; (f) just before CCB ended; (g) 60 minutes after CCB; and (h) 24 hours after CCB. All patients had CCB with a bubble oxygenator, centrifugal pump, and arterial line filter and were cooled to 26 °C to 28 °C during the procedure. Cold crystalloid potassium cardioplegia was used. Blood aspirated from the operative field was diluted in saline and centrifuged in a Dideco cell sorter (Mirondola, Italy) at 2,800 g and infused into each patient as packed cells.

Blood samples were collected into 1/10 volume sodium citrate (final concentration 0.38%) containing PGE1 (final concentration 0.3 μmol/L) to prevent in vitro cell activation. Three milliliters were prepared as described previously for βTG measurements. Twelve milliliters were centrifuged at 2,225 g for 15 minutes at 23 °C. The supernatant was then recentrifuged at 27,300 g for 15 minutes and the resulting supernatant frozen at −70 °C in 0.8-mL aliquots for subsequent measurements of lactoferrin and HNE.

Simulated extracorporeal circulation. Perfusion circuits were assembled from standard silicone rubber components, polycarbonate connectors, and spiral coil membrane oxygenators as described previously. Blood and gas compartments were flushed with 100% carbon dioxide for 15 minutes prior to priming the circuit. Oxygenators were primed by applying a vacuum to the lower gas port. Five hundred milliliters of blood were drawn from random donors who had abstained from any medications for 2 weeks directly into circuit blood and gas compartments. The blood was oxygenated with 0.3 μmol/L to prevent in vitro cell activation. Three milliliters were added; plates were incubated for 40 to 60 minutes, and the absorbance was measured at 450 nm.

ELISA for lactoferrin. Plasma lactoferrin levels were measured as described previously from this laboratory.

Neutrophil isolation and aggregation. Neutrophils were isolated and aggregation was measured with a platelet aggregometer (Chronolog, Havertown, PA) as described previously from this laboratory. Neutrophils (1 × 10⁷/mL) were preincubated for 10 minutes at 37 °C with either PGE1 (0.3 μmol/L) or lidocaine (100 μmol/L). They were then challenged with the chemotactic peptide FMLP (100 nM) and extent of aggregation measured. The results were compared to neutrophils that had not been exposed to either drug.

Lidocaine measurements. Plasma levels of lidocaine were measured at 0, 2, 10, 60, and 120 minutes after the start of recirculation, employing an immunoassay lidocaine kit obtained from SYVA, Palo Alto, CA.

Statistical analysis. Student's t test (paired) was used to test for significant differences between treatments at the end of SECC at 120 minutes. In all cases +/− values stated indicate the mean ± the SEM. Analysis of variance was performed using all values for the data on the last three figures. All values listed as significant have a less than 5% occurrence by chance alone.

RESULTS

Clinical cardiopulmonary bypass. Following heparin administration, prebypass platelet counts fell to 92% ± 6% of the initial platelet level. By five minutes into bypass, platelet counts had decreased to 40% ± 4% (Fig 1) and remained at baseline levels for the duration of bypass. However, plasma βTG rose throughout CCB (Fig 1).

After heparin administration, prebypass neutrophil counts fell to 90% ± 4% of the initial level and by 45 minutes neutrophil counts dropped to 67% ± 7%. However, by 60 minutes postbypass the neutrophil counts had returned to 103% ± 6% of the initial neutrophil level (Fig 2). Prebypass
Lactoferrin levels rose from a baseline of 0.06 ± 0.02 µg/mL to 0.56 ± 0.17 µg/mL before bypass. During CCB, lactoferrin levels continued to rise steadily to a maximum of 1.66 ± 0.33 µg/mL just before the end of CCB. However, lactoferrin concentrations fell to 1.45 ± 0.48 µg/mL 60 minutes after bypass and by 24 hours had returned to an initial prebypass level of 0.23 ± 0.06 µg/mL (Fig 2). In contrast, the prebypass levels of HNE did not change. After five minutes of bypass, HNE levels began to increase from a baseline of 0.49 ± 0.07 µg/mL. HNE levels rose throughout bypass to 1.65 ± 0.68 µg/mL. By 60 minutes, the HNE was still maximal at 1.71 ± 0.55 µg/mL but returned to a value of 0.71 ± 0.07 µg/mL by 24 hours after bypass (Fig 2).

Simulated extracorporeal circulation. During recirculation the circulating platelet count declined within two minutes and slowly increased over the next two hours, as has been reported previously. A steady rise in plasma levels of βTG to 5.13 µg/mL at 120 minutes (Fig 3) was observed. Addition of PGE\(_1\) (0.3 µmol/L) completely preserved the prebypass platelet counts and prevented βTG release (Fig 3), while lidocaine (100 µmol/L) showed no effect on the release of βTG (5.66 µg/mL) at 120 minutes (Fig 3).

During SECC, neutrophil counts declined after 30 minutes, but even at 120 minutes the value was 81% ± 21% of baseline. Neither PGE\(_1\) (0.3 µmol/L) nor lidocaine (100 µmol/L) resulted in significant preservation of circulating neutrophil counts (Fig 4).

During SECC there was a steady rise in the plasma levels of lactoferrin from a baseline of 0.13 ± 0.04 µg/mL to 1.62 ± 0.22 µg/mL at 120 minutes. This level of lactoferrin at 120 minutes differed significantly from the level of 0.26 ± 0.13 µg/mL (P < .05) found in the control maintained at 37 °C for 120 minutes without exposure to the circuit. PGE\(_1\) (0.3 µmol/L) decreased the rate but did not completely inhibit lactoferrin released from neutrophil-specific granules (Fig 5). Lidocaine (100 µmol/L) appeared to show an inhibitory effect on the release of lactoferrin for the initial 20 minutes of perfusion (Fig 5). Although the t\(_1/2\) of PGE\(_1\) is known to be several hours in plasma, the t\(_1/2\) of lidocaine has not been previously examined. To estimate how much lidocaine was present in our circuit, we measured lidocaine levels in our samples and determined the t\(_1/2\) of its disappearance to be 7 minutes. Since lidocaine was only introduced at the beginning of SECC, lidocaine levels would be 12% of that of the initial concentration at 21 minutes.

During recirculation there was a steady rise in plasma levels of HNE from a baseline of 0.05 ± 0.06 µg/mL at 1.86 ± 0.41 µg/mL at 120 minutes. This level of HNE when analyzed at 120 minutes differed significantly from the level of 0.37 ± 0.06 µg/mL HNE (P < .05) found in the 120-minute standing control not exposed to the circuit. In contrast to the effect on either neutrophil count or lactoferrin...
release, analysis of variance indicates a significant inhibition of the release of HNE during recirculation by both PGE, and lidocaine respectively (Fig 6).

To ascertain whether the inhibition of neutrophil degranulation observed in SECC was due to a direct effect on the cells, neutrophils were preincubated for ten minutes at 37 °C with either PGE, (0.3 μmol/L) or lidocaine (100 μmol/L). Both treatments inhibited the maximal extent of in vitro aggregation induced by the chemotactic peptide FMLP by 50% and 53%, respectively (data not shown).

**DISCUSSION**

Cardiopulmonary bypass is used in approximately 200,000 Americans of all ages each year. The procedure differs substantially from hemodialysis in that the entire cardiac output in CCB passes through multiple components of the perfusion system, whereas in hemodialysis only a small (5% to 10%) portion passes through. The procedure is technically complex and varies in detail from patient to patient. With rare exception the patient's blood is diluted with crystalloid solutions yielding hematocrits of 20 to 25, cooled to 26 °C to 28 °C, and continuously filtered, washed, centrifuged, and returned to the patient as packed RBCs. In addition, patients receive a wide variety of drugs, anesthetic agents, and occasional blood transfusions. Usually the heart is perfused with cold cardioplegic solution every 20 to 30 minutes, and this solution (typically totalling 1 to 2 L) returns to the perfusate. The extensive dilution and processing of autologous blood and the presence of the patient's metabolic and clearance mechanisms, frustrate attempts to completely define all variables of blood-surface interaction in the clinical setting. However, because of possible clinical significance, it is important to verify the appearance and disappearance of various blood elements and cell-specific markers in vivo. Thus, this study for the first time identifies the appearance of lactoferrin and HNE in the circulation of patients who have undergone open-heart surgery.

SECC with fresh heparinized human blood permits qualitative measurements and careful study of the blood elements that directly interact with synthetic surfaces of the cardiopulmonary bypass system. Many of the variables that complicate the interpretation of in vivo results are eliminated. The use of heparin does not pose a problem with regard to neutrophil activation in SECC, since there is no significant rise in plasma levels of lactoferrin and HNE in 120-minute standing control samples that have not been exposed to the circuit. This model has proved particularly valuable in studies of platelet-surface interactions, and has led to measures to protect platelets during CCB.

CCB activates platelets and complement, but heretofore activation of neutrophils with concomitant release of neutrophil-specific proteins has not been definitively demonstrated. Several investigators have demonstrated sequestration of neutrophils within capillaries during and immediately after cardiopulmonary bypass. Appearance of both the neutrophil-specific granule protein lactoferrin and azurophilic granule enzyme HNE in the plasma of both CCB and SECC indicates that neutrophils are stimulated and degranulation occurs.

Neutrophils contain many powerful lysosomal hydrolases as well as significant amounts of HNE, a major granular protease. Since the extracellular environment has a neu-
central pH, released HNE has a potential for catalytic activation and may be important in mediating complications of CCB. HNE is a potent fibrinogenolytic and fibrinolytic enzyme,26 that alters other coagulation proteinases.27,28 hydrolyzes platelet glycoprotein Ib,29 reduces the number of thrombin-binding sites per platelet,30 and exposes fibrinogen receptors on the platelet surface.31 Thus, HNE present in the extracellular environment after neutrophil degranulation could contribute to the hemorrhagic complications of prolonged CCB. In addition, because HNE attacks lung parenchyma32 and produces endothelial injury,33 it may play a role in the capillary, pulmonary, and compartmental fluid changes associated with CCB. These changes could occur at HNE concentrations found in this study (Fig 2). When alpha-1-proteinase inhibitor is present, rapid inactivation of HNE occurs,34 but oxidation of the critical Methionine residue of this plasma protease inhibitor may result in its inactivation allowing active HNE to act on connective tissues or other substrates.

Lactoferrin, located in the specific granules, is released at shorter incubation times and/or lower concentration of agonists than HNE.35 It may be more difficult to inhibit lactoferrin exocytosis than HNE release. Lactoferrin may function as an autacoid potentiating other neutrophil agonists.36 SECC allows evaluation of agents that may modulate the ability of leukocytes and platelets to extrude their intracellular proteins. Lidocaine (100 μmol/L), a known inhibitor of neutrophils7 with a 1/2 of seven minutes in this system, inhibits the release of lactoferrin for the first 20 minutes of recirculation, and then as the effect of lidocaine begins to wear off, lactoferrin gradually increases to levels seen in the absence of the drug (Fig 5). The concentration of lidocaine that has been used as an antiarrhythmic agent is 5 to 20 μmol/L.37 In contrast, lidocaine virtually prevents the appearance of HNE (Fig 6) during SECC since lower concentrations are required for this effect. The effect of lidocaine is probably mediated directly since this compound inhibits neutrophil aggregation in vitro. Since we have not yet determined the minimal concentration necessary for inhibition of neutrophil exocytosis, its potential as a chemical inhibitor of neutrophil granule release cannot be ruled out. In contrast, βTG levels are not altered by lidocaine (Fig 3).

PGE1 (0.3 μmol/L), a known inhibitor of platelet function,19 not only preserves the percent of initial platelet level and inhibits the release of βTG (Fig 3) as observed previously7 but also affects neutrophil degranulation as reflected by the attenuated release of lactoferrin (Fig 5) and total inhibition of HNE degranulation (Fig 6) during SECC. PGE1 has already been shown to inhibit both leukocyte aggregation in the pulmonary microcirculation18 and granulocyte function.39 We now demonstrate that PGE1 directly inhibits FMLP-induced neutrophil aggregation, in agreement with the observation of Fantone et al40 that PGE1 decreased the generation of superoxide anion production and lysosomal enzyme secretion of neutrophils in response to FMLP. Additionally, recent work by Holcroft et al41 describes the ability of PGE1 to inhibit inflammation in the lungs of patients with adult respiratory distress syndrome.

Although the percent of initial platelet levels during both SECC19 and CCB (Fig 1) decreases markedly, the percent of initial neutrophil levels falls to a lesser degree in either SECC (Fig 4) or CCB (Fig 2). Platelets adhere to synthetic surfaces of extracorporeal perfusion system1,38 and are activated to synthesize thromboxanes4 and release α-granule contents.5 Membrane α1-adrenergic4 and fibrinogen receptors5 are reduced, and remaining circulating platelets lose their ability to aggregate in response to established agonists.6 In contrast, the failure of neutrophil counts to decrease to the extent of that of the platelets during recirculation suggests that neutrophil adherence to the synthetic surfaces of the system is less sensitive as an activating mechanism than it is with platelets. Thus, the soluble neutrophil agonists that are generated as a result of activation of other blood elements and/or proteins on the surface of the extracorporeal circuit are implicated in neutrophil activation.

Complement activation occurs during CCB.24 The formation of C5a, a potent neutrophil agonist,42 is inferred from observed increases in C3a.43 No direct increase of C5a has as yet been measured, probably because any C5a generated is readily internalized by the neutrophil. Thus, the contribution of C5a as a neutrophil agonist is not yet clear in SECC.

Recent studies by Heller et al44 have indicated that during CCB plasma prekallikrein levels significantly decrease, and the generation of kallikrein-like activity has been observed. Plasma kallikrein has been shown to induce neutrophil chemotaxis,45 aerobic glycolysis,46 aggregation,47,48 and degranulation17,46 and may serve as a direct agonist in this system. Additionally, kallikrein activates Factor XII to Factor XIIa,49,50 which recently has been shown to aggregate and stimulate exocytosis of HNE from neutrophils.18 Very little is known about the contact system enzymes during CCB, and further investigation is needed.

In this study we have observed increased plasma levels of βTG in both SECC (Fig 3) and CCB (Fig 1). Platelet-specific proteins such as βTG or platelet-derived growth factor may serve as potential neutrophil agonists. Recent studies indicate that other platelet-specific α-granule proteins, platelet Factor 451 and platelet-derived growth factor induce neutrophil chemotaxis.52 In addition, the formation of leukotrienes, potent autacoids for neutrophils, may also play a role in this activation process. Any or all of the aforementioned neutrophil agonists could singularly or in concert at lower concentrations be responsible for the observed neutrophil activation and subsequent degranulation that we have documented to occur during both SECC and CCB. Identification of the responsible humoral substances would allow for rational and specific intervention during CCB to prevent neutrophil alterations and their potential deleterious consequences during CCB.

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