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Human platelets efficiently kill IgG-opsonized *E. coli*

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Abstract

Platelets are known contributors of hemostasis but have recently been shown to be important in inflammation and infectious diseases. Moreover, thrombocytopenia is often observed in patients with sepsis. We previously reported that platelets actively phagocytosed IgG-coated latex beads. In the current study, the capacity of human platelets to participate in host defense against bacterial infections was determined by assessing their ability to kill *E. coli*. Washed human platelets were incubated with unopsonized or IgG-opsonized *E. coli* and evaluated for binding and killing of *E. coli*. We found that although both unopsonized and IgG-opsonized *E. coli* associated with platelets, only IgG-opsonized *E. coli* were efficiently killed unless platelets were activated by a potent agonist. The bactericidal activity was dependent on Fc γ RIIA, was sensitive to cytochalasin D, but was not due to reactive oxygen metabolites. These data suggest that platelets may play an important role in protection against infection.

Keywords

Fc γ RIIA; IgG; *E. coli*; platelet

Introduction

Platelets are recognized for their critical role in maintaining hemostasis. More recently, additional activities are being attributed to platelets including participation in wound repair, angiogenesis, inflammation, and even host defense against infection (reviewed in (17)). For example, it is known that patients with sepsis often present with thrombocytopenia, which may be due to disseminated intravascular coagulation stimulated by bacterial products (6). Furthermore, platelets have been observed to interact with various bacterial species *in vitro* (reviewed in (4)). These interactions require various platelet surface proteins, including toll-like receptors, fibrinogen receptors and Fc γ RIIA(2, 5). Fc γ RIIA is the only IgG receptor present on human platelet surfaces and is responsible for binding and internalization of IgG-containing targets (1, 8, 23). Specifically, we have recently shown that platelets are capable of binding and internalizing IgG-coated latex beads, and the internalization is dependent on Fc γ RIIA and actin polymerization, similar to phagocytosis observed in leukocytes (1). Furthermore, others have observed platelets with internalized bacteria, viruses, liposomes, erythrocyte fragments and latex particles (4, 7, 12, 15, 26). Additional evidence for the bactericidal potential of platelets comes from studies examining platelet lysates which contain platelet microbicidal proteins (PMP) and β -defensin, both capable of killing *Staphylococcus aureus* (11, 24, 25). In light of the previous findings, the current study was

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designed to evaluate the ability of platelets to bind and kill bacterium, and the potential role of Fc γ RIIA in this process.

Materials and Methods

Platelet Preparation

Human blood from healthy volunteers was obtained via venipuncture consistent with the University of Toledo Biomedical Institutional Review Board policy. Blood was collected in Vacutainer[®] (BD, Franklin Lakes, NJ) tubes containing anticoagulant: 22.0 g/L trisodium citrate, 8.0 g/L citric acid, and 24.5 g/L dextrose. The tubes were immediately centrifuged for 10 minutes at $200 \times G$ to obtain platelet-rich plasma (PRP). The PRP was washed two times with pH 6.5 tyrode buffer containing: 2.75 g/L trisodium citrate, 1.0 g/L citric acid, 3.125 g/L dextrose, and 8.5 g/L sodium chloride. The solution was centrifuged after each wash for 10 minutes at $440 \times G$. After the second wash, the platelets were resuspended in 500 μ L of pH 7.4 tyrode buffer containing: 8.0 g/L sodium chloride, 0.2 g/L potassium chloride, 0.2 g/L magnesium chloride, 0.45 g/L sodium phosphate dibasic, and 0.9 g/L HEPES. Platelets were counted using a hemocytometer.

Bacteria Preparation

EGFP-expressing *E. coli* K12 (generously provided by Dr. Robert Blumenthal), were stored in glycerol stock at -80°C until use. Bacteria were grown overnight at 37°C in 10 mL of LB Miller's broth (Difco, Detroit, MI). Bacteria was centrifuged at $4000 \times G$ for 10 minutes then resuspended in 2 mL of Dulbecco's Phosphate Buffered Saline (DPBS) buffer (Thermo Fisher Scientific, Waltham, MA) without calcium or magnesium. Some samples were then opsonized for 30 min at 37°C with goat anti-*E. coli* polyclonal IgG (abcam[®], Cambridge, MA). Bacteria were washed twice with DPBS and counted using a hemocytometer.

Bacterial Killing Assay

Unstimulated platelets or platelets ($1 \times 10^7/100 \mu\text{L}$) activated with 0.1 unit/ml thrombin (Chronolog,) and *E. coli* were mixed at a Multiplicity of Infection (MOI) of 1 then placed into a 12-well tissue culture plate (Corning), then centrifuged ($300 \times G$ for 60 sec) to induce contact. The plate was then floated on a 37°C water bath for 60 minutes before adding a 1% saponin solution to lyse platelets that had phagocytosed *E. coli*. Solutions were then serially diluted and plated on LB agar plates, incubated overnight at 37°C and CFU were determined for each plate. For inhibitor experiments, platelets were pre-treated with the inhibitor for 15 min at 37°C prior to adding bacteria. Cytochalasin D (Sigma Aldrich, St. Louis, MO) was used at a final concentration of 20 μM , anti-Fc γ RIIA mAb (clone IV.3, purified from hybridoma) was used at 20 $\mu\text{g}/\text{mL}$, and diphenyleneiodonium chloride (DPI)(Sigma Aldrich) was used at 100nM. Each individual experiment was performed in triplicate wells and serial dilutions were plated in triplicate. Each experiment was performed at least three times.

Fluorescence Microscopy

Platelets and *E. coli* were mixed at an MOI of 1 and plated in 12-well tissue culture plates, as stated above. After incubation for 60 min at 37°C , the bacteria were stained using the Live-Dead BacLight bacterial viability kit (Invitrogen, Carlsbad, CA) and viewed using epifluorescence microscopy (Leica DMIRB, Leica Microsystems, Buffalo Grove, IL). Images were acquired by a QICAM Fast 1394 (Q-imaging, Surrey BC, Canada) and images were processed using Q-capture Pro software.

Statistical Analysis

Significance of bactericidal activity compared between two groups was assessed using two-tailed unpaired student's t-test. Data shown are mean $\pm$ SE. A value of $p < 0.05$ was considered significant.

Results

Previous reports from our group and others suggest that platelets can bind and internalize various targets of similar size to bacteria (1, 5, 7, 14, 19, 26). In order to determine if platelets bind *E. coli* we incubated freshly washed human platelets with bacteria and assessed viability using the Live Dead BacLight kit and epifluorescence illumination. We observed that platelets were bound to both unopsonized and IgG-opsonized *E. coli* (Fig. 1A). These data suggest that *E. coli* can be bound by platelets independent of plasma or serum proteins. Surprisingly, IgG-opsonization of *E. coli* did not appear to increase platelet binding as we observed similar numbers of bacteria per field of view (Figure 1B). Moreover, very few unopsonized *E. coli* were found to be stained with propidium iodide, suggesting that platelets failed to kill the bacteria even though they bound them. However, a slight (but statistically insignificant) decrease in bacterial viability was observed when *E. coli* were opsonized with IgG (Fig. 1B).

As shown in Fig. 1B, experimental variability was considerable so we employed a more consistent assay to quantify bactericidal activity by measuring viable bacteria colony forming units (CFU) after exposure to platelets. After incubating platelets with unopsonized or IgG-opsonized *E. coli*, platelets were lysed and supernatants were serially diluted, plated on LB agar plates and the CFUs/ml were enumerated. Platelets incubated with unopsonized *E. coli* did not display any bactericidal activity (Fig. 2A). However, *E. coli* opsonized with IgG were efficiently killed (Fig. 2B). Because platelets only displayed bactericidal activity toward IgG-opsonized *E. coli*, we determined if this activity is attributed to the platelet IgG receptor, Fc γ RIIA. To determine if killing of IgG-opsonized *E. coli* is Fc γ RIIA dependent, platelets were pre-treated with a blocking anti-Fc γ RIIA mAb (clone IV.3) or an isotype control IgG. Killing of IgG-opsonized *E. coli* was inhibited in the presence of the Fc γ RIIA blocking antibody, but not isotype-control IgG (Fig. 2B). Bactericidal activity of Fc γ RIIA on traditional leukocyte populations involves phagocytosis, and we have shown that platelet Fc γ RIIA can mediate phagocytosis of IgG-coated beads that can be inhibited by cytochalasin D (1). Incubating platelets with cytochalasin D prior to adding IgG-opsonized bacteria produced a significant decrease in bactericidal activity, suggesting that phagocytosis is necessary for IgG-mediated killing. Since leukocyte bactericidal activity is also associated with the production of reactive oxygen metabolites (ROM), and platelets have been shown to produce significant amounts of ROM, we tested the effect of the ROM inhibitor, DPI, on bactericidal activity in platelets (13, 22). Surprisingly, DPI had no effect on bacterial killing. These data suggest that human platelets are capable of bactericidal activity that is dependent on Fc γ RIIA and actin, but does not require ROMs.

Fc γ RIIA is known to induce platelet activation upon binding of IgG-coated latex beads (1). The activation leads to release of significant amounts of sCD40L and RANTES, expression of surface markers indicative of platelet activation (CD62P), and release of PMPs (1, 18). One possibility for the difference in killing between IgG-opsonized and non-opsonized *E. coli* could be the activation status of the platelets. To test this, the bactericidal activity of activated platelets was tested against IgG-opsonized and non-opsonized *E. coli*. Interestingly, activation of platelets with 0.1 unit/ml of thrombin (a potent platelet activating agent) resulted in the ability to kill non-opsonized *E. coli* while it had no significant additive effect on IgG-opsonized *E. coli* (Fig. 3). Of particular note, IgG-opsonized *E. coli* were killed more efficiently than non-opsonized *E. coli* in the presence of activated platelets.

Therefore, we interpret this to mean that bactericidal activity is dependent on platelet activation which can be induced by either ligation of Fc γ RIIA or by thrombotic agents.

Discussion

Due to the strong evidence that platelets are capable of binding and internalizing particles similar in size to bacteria, and that platelets produce both ROM and PMP, we tested the hypothesis that platelets could kill bacteria, using a non-pathogenic laboratory strain of *E. coli*. We observed that platelets could bind *E. coli* in the absence and presence of opsonizing IgG, but that only IgG-opsonized *E. coli* were efficiently killed in a Fc γ RIIA-dependent manner. These observations imply that platelet-binding of non-opsonized *E. coli* provides insufficient signals to elicit bactericidal activity by platelets. Most studies of platelet-bacterial interactions have found that plasma proteins (fibrinogen, collagen, von Willebrand factor) are required for binding with platelet surface proteins (α IIB β 3, GPVI, and GPIb, respectively) and these induce varying degrees of activation (4). In our studies, we observed *E. coli* binding in the absence of any plasma proteins. Although platelets express TLR4, which could mediate binding of Gram-negative bacteria, platelets do not express essential TLR4 binding partners CD14 or MD-2 (and our experiments were performed in PBS), so TLR4-mediated interaction is not likely. Platelets also express many other TLR family-members as well as scavenger receptors (CD36) which may participate in binding (3, 10, 16, 21, 27).

Our data also suggest that bactericidal activity is dependent on actin rearrangement, since the addition of cytochalasin D was able to block killing. In standard leukocyte assays, inhibition of actin rearrangement is interpreted as inhibiting phagocytosis. We have shown that treating platelets with cytochalasin D inhibits phagocytosis of IgG-opsonized latex beads (1). However, actin rearrangement is also involved in fusion of intracellular granules with the plasma membrane in platelets. Therefore, the ability of cytochalasin D to inhibit killing could also be due to blocking the secretion of granule contents, which may contain a number of microbicidal proteins (18). Of particular interest is the observation that bactericidal activity appears to be independent of ROM. Although platelets are known to produce ROM, the quantity of ROM produced may be insufficient to reach the concentration necessary to kill *E. coli* at an MOI of 1(22).

Previous reports suggest that platelets can kill *S. aureus* but have marginal bactericidal activity at a MOI=1, which is consistent with our observations (20). However, when the ratio of platelets to *S. aureus* is increased to 1000 or 10,000:1, platelets are able to kill nearly 100% of *S. aureus* and the killing is dependent on release of PMP after stimulation of adenine nucleotide receptors (P2X₁ and P2Y₁₂)(20). In addition to microbicidal proteins stored in granules, platelets also express β -defensin, which has recently been reported to be localized in the cytoplasm and is potent at killing *S. aureus* (11). Importantly, the β -defensin mediated killing is dependent on the production of *S. aureus* α -toxin which presumably permeabilizes platelets thus and causing β -defensin release (11). These data suggest that *S. aureus* killing most likely takes place independent of internalization and favors extracellular killing even though *S. aureus* has been reported to be phagocytosed by human platelets (26). Important to our studies, laboratory strains of *E. coli* (K12) (including the one we used) have not been reported to express any pore-forming molecules such as α -toxin (although some pathogenic strains express lytic molecules)(9).

Although platelets have been reported to internalize many different particles, the ability to kill bacteria is only now becoming appreciated. We report here that platelets can kill laboratory strains of *E. coli*. Interestingly, we did not observe significant differences in bactericidal activity using different MOIs or longer incubations times up to two hours (data

not shown). However, killing appears to be dependent on activation induced by either ligating Fc γ RIIA or by thrombotic stimulation. We interpret these observations to mean that platelets can kill bacteria via internalization in a Fc γ RIIA-dependent manner but the majority of killing occurs by the release of PMPs from granules upon activation. However, further investigation is necessary to elucidate the bactericidal mechanism(s).

Conclusion

Human platelets can bind *E. coli* in the presence and absence of opsonizing IgG. Human platelets also have the potential to kill IgG-opsonized *E. coli* in a Fc γ RIIA-dependent and actin-dependent fashion.

Acknowledgments

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Figure 1. Association of *E. coli* with human platelets

Washed human platelets were incubated with *E. coli* K12 strain at a MOI of 1 for 60 minutes. **A:** Plates were washed, stained with BacLight Live-Dead stain (Syto 9 and propidium iodide) and imaged using epifluorescence microscopy. Representative PI-positive *E. coli* are indicated by arrows and PI-negative *E. coli* by arrow-heads. **B:** Numbers of platelet-bound *E. coli* per field of view were counted and the % PI-negative *E. coli* was calculated. * p<0.05

Figure 2.

Human platelet killing of *E. coli*. **A:** Washed human platelets were incubated with *E. coli* K12 strain at a MOI=1 for 60 minutes at 37°C in triplicate wells of 12-well plates. Platelets were lysed and samples were serially diluted and plated on duplicate LB agar, incubated overnight at 37°C and colony forming units were counted and expressed as CFU/ml. **B:** Experiment performed exactly as in (A) except that *E. coli* were opsonized with IgG. Bars are mean $\pm$ SE (n=3). * p<0.05

Figure 3.

Killing of *E. coli* by activated platelets. IgG-opsonized or non-opsonized *E. coli* were incubated with 0.1 unit/ml thrombin in the presence or absence of platelets for 60 minutes at 37°C in triplicate wells of 12-well plates. Platelets were lysed and samples were serially diluted and plated on LB agar, incubated overnight at 37°C and colony forming units were counted and expressed as CFU/ml. Bars are mean $\pm$ SE (n=3). * p<0.05 NS=not significant