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Human Platelet FcγRIIA and Phagocytes in Immune-Complex Clearance

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Abstract

In addition to their primary role in hemostasis and wound healing, platelets play important roles in a multitude of physiological functions including immune and inflammatory responses. We present data that platelets, by virtue of their expression of the human specific FcγR, FcγRIIA, bind IgG complexes *in vivo* and that circulating phagocytes from healthy individuals internalize platelets *in vivo*. Human platelets, as a consequence of their expression of FcγRIIA, may thus contribute to the clearance of IgG-containing complexes from the circulation.

Keywords

platelets; immune-complexes; Fcγ receptor; phagocytosis; phagocytes; transgenic mice

Introduction

Although platelets are best known as the primary mediators of hemostasis in humans and other mammals, they also play important roles in a multitude of other physiological functions (Klinger and Jelkmann, 2002). Platelets are rapidly deployed to sites of injury or infection and release proteins that kill certain bacteria and fungi. Platelets also modulate inflammatory processes by interacting with leukocytes and by secreting cytokines, chemokines, and other inflammatory mediators (Elzey et al., 2005; Semple and Freedman, 2010). In addition, their function has been linked with various pathological conditions, including atherosclerosis, arthritis and immune thrombocytopenic purpura (ITP) (Semple and Freedman, 2010; Cines and Blanchette, 2002; Gasparyan, 2010). Our demonstration that human platelets can bind and endocytose IgG complexes *in vitro* (Worth et al., 2006) suggested that platelets also participate in the clearance of IgG-containing complexes.

Immune complexes (IC) are present in the circulation of healthy individuals and the formation of such complexes is part of a normal immune process. Efficient clearance of IgG complexes can be critical because their deposition in tissues and organs can set off reactions that lead to inflammation and tissue damage (Jancar and Sánchez Crespo, 2005; Mayadas et

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al., 2009). For example, in some pathological conditions, including autoimmune diseases such as systemic lupus erythematosus and autoimmune glomerulonephritis significant amounts of immune complexes are formed and deposited in the kidney and other tissues, causing severe injury (Niederer et al., 2010; Bagavant and Fu, 2009).

Fc γ RIIA is the only Fc γ receptor expressed on platelets (Cassel et al., 1993; King et al., 1990). On professional human phagocytes such as monocytes and neutrophils, Fc γ RIIA plays an important role in the clearance of IgG immune complexes and the phagocytosis of IgG coated particles (McKenzie and Schreiber, 1994). Apart from the recognition that Fc γ RIIA is important for platelet activation by von Willebrand factor (Canobbio et al., 2001), the role of Fc γ RIIA in platelet function has not been well defined.

It is known that platelets from patients suffering from certain autoimmune and thrombocytopenic disorders have high levels of surface bound IgG. Platelets from normal donors also bind IgG immune complexes *in vivo*, albeit at lower levels (Romero-Guzmán et al., 2000; George, 1990; Christopoulos et al., 1993; Court et al., 1987). We present evidence that platelets of normal individuals can serve as vehicles for delivery of immune complexes for destruction by phagocytes and that circulating neutrophils/ monocytes play a role in this process. We also demonstrate that in addition to the ability to ingest small IgG particulates (heat aggregated IgG, Worth et al., 2006), human platelets can internalize larger IgG coated particles by a process similar in some respects to phagocytosis in leukocytes.

Materials and Methods

Reagents

PE labeled F(ab')₂ fractions of goat anti-rat IgG, goat anti-human IgG, goat anti-mouse IgG and FITC labeled human IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Mouse anti-human CD61 was obtained from BD Biosciences, (San Jose, CA). Fab fractions of mAb anti-human Fc γ RII (IV.3) were prepared in our laboratory. Monomeric human IgG (mono-IgG) was prepared by ultracentrifugation of human IgG (MP Biomedicals, OH) at 80K for 15 min. Heat aggregated human IgG (HA-IgG) was prepared by heating human IgG (10mg/ml) in PBS at 62°C for 20 min, followed by centrifugation at 12,000 rpm for 10 min to remove insoluble aggregates. The final IgG complex was used at 100 μ g/ml as assessed by absorbance at 280 nm.

Animals and cell lines

Fc γ RIIA transgenic mice were provided by Dr. Steven E. McKenzie (Thomas Jefferson University, Philadelphia, PA). All protocols were performed in accordance with National Institutes of Health guidelines and with the approval by the University of Pennsylvania Animal Use Committee. COS cells stably expressing Fc γ RIIA cell line (COSIIA) was constructed in our laboratory as previously described (Huang et al., 2004).

Isolation of human and mouse platelets

Platelet-rich plasma was prepared from heparinized venous blood of healthy volunteers by centrifugation of the blood at 900 rpm (175 \times g) at room temperature. Platelets were isolated by gel filtration on Sepharose 2B. A similar protocol was used to isolate platelets from WT and Fc γ RIIA TG mice.

Internalization of IgG coated beads

Streptavidin conjugated polystyrene beads (0.1, 0.5 and 1.0 μ m) (Bangs Laboratories, Inc, Fishers, IN) were coated with biotin/anti-biotin mAb according to the manufacturer's instructions. The IgG-coated beads were allowed to bind (45 min) to platelets at 4°C. One

set of cells was maintained at 4°C as a binding control. Other cells were warmed to 37°C for 20 min to allow internalization of bound IgG-coated beads and then returned to 4°C to stop internalization. Goat anti-mouse IgG conjugated to phycoerythrin (PE) was added to label remaining externally bound IC polystyrene beads and cell fluorescence was analyzed by flow cytometry. The cells maintained at 4°C retain a large amount of IC on the surface and display bright fluorescence. However, cells incubated at 37°C that have internalized some of the surface bound IgG-coated beads display less fluorescence. Internalization is defined as the % loss of surface expression (fluorescent label). For each condition, one set of cells was pre-incubated with cytochalasin D (CytD) and CytD was also present during the uptake assay.

Isolation of human monocytes and neutrophils

Heparinized venous blood collected from healthy volunteers was overlaid on Ficoll-Hypaque (LSM; Organon-Teknika, West Chester, PA) and centrifuged at 2200 rpm for 30 minutes. Pelleted granulocytes were cleared of residual erythrocytes by brief hypertonic lysis and resuspended in RPMI. Mononuclear cells from the interface were washed, suspended in RPMI medium (GIBCO, Grand Island, NY) plus 10% heat inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT), and incubated at 37°C on serum-coated flasks for 45 minutes to adhere monocytes. Nonadherent cells were removed by washing with RPMI.

Internalization of human platelets by human monocytes and neutrophils: Analysis by flow cytometry

Gel filtered human platelets were incubated with HA-IgG for 30 min at 0°C. The IgG-coated platelets were washed and then incubated with human monocytes or neutrophils on ice for 45 min. One aliquot was maintained at 0°C for an additional 30 min for use as a binding control. The other aliquot was rapidly warmed and incubated at 37°C for 30 min to allow internalization of the HA-IgG platelet complexes. Both sets of cells were washed with ice-cold PBS and further incubated for 30 min on ice with 3.5 µg/ml phycoerythrin (PE) conjugated F(ab')₂ goat anti-human IgG to label surface bound HA-IgG platelet complexes. Cells were washed again with ice-cold PBS before analysis by flow cytometry. The mean fluorescence (MF) of cells maintained on ice throughout the procedure was used as the binding reference (no phagocytosis) (Van de Winkel and Capel, 1993). In other experiments, monocytes were exposed to platelets labeled with FITC-conjugated HA-IgG. Under these conditions, FITC fluorescence represents both surface-bound and intracellular HA-IgG platelet complexes.

Electron microscopy

Monocytes, isolated as described above, were washed and fixed with 2% glutaraldehyde at 4°C overnight. The samples were then washed extensively with cacodylate buffer, fixed with osmium tetroxide, dehydrated, embedded in Spurr's resin and sectioned (Worth, 2006). The sections were viewed at the EM core facility at University of Pennsylvania.

Results and Discussion

Human platelets from healthy donors bind IgG *in vivo*

The binding of IgG to the surface of normal human platelets *in vivo* is here illustrated using flow cytometry (Figure 1A). Gel filtered human platelets were treated with fluorescence-labeled antibody directed to human IgG [PE-labelled F(ab')₂ anti-human IgG]. The large increase in fluorescence intensity of these platelets compared to platelets treated with control antibody (PE labelled F(ab')₂ anti-rat IgG) or saline indicates the presence of surface bound human IgG.

It is known that Fc γ RIIA, the only Fc γ R expressed on platelets, binds IgG complexes (McKenzie and Schreiber, 1994). Since platelet Fc γ RIIA encounters circulating monomeric IgG as well as IgG complexes *in vivo*, we examined whether platelet Fc γ RIIA can bind IgG complexes in the presence of physiologic levels of monomeric IgG (Figure 1B). The mean fluorescence intensity of platelets treated with monomeric IgG (10mg/ml) before exposure to heat-aggregated IgG (HA-IgG) is equivalent to that of platelets pretreated with saline, indicating that monomeric IgG does not interfere with the binding of HA-IgG complexes to platelet Fc γ RIIA. Figure 1B also demonstrates that incubation of the platelets with mAb IV.3 (which binds to the ligand binding site of Fc γ RIIA) before exposure to human HA-IgG diminishes fluorescence intensity, illustrating that access to Fc γ RIIA is required for IgG complex binding to platelets.

The inability of monomeric IgG to interfere with platelet Fc γ RIIA binding to IgG complexes is a consequence of the low affinity of Fc γ RIIA for monomeric IgG and its high affinity for complexed IgG (Van de Winkel and Capel, 1993). In Figure 1C, we illustrate that Fc γ RIIA binds only aggregated IgG and not monomeric IgG, using as model COS cells transfected to stably express Fc γ RIIA (COS IIA). Flow cytometry using PE-tagged F(ab')₂ goat anti-human IgG detected only aggregated IgG on the cell surface; i.e. there was a substantial increase in fluorescence intensity in cells treated with HA-IgG human IgG but the mean fluorescence intensity of cells treated with monomeric human IgG (10mg/ml) followed the same pattern as control cells treated with saline.

Because mice do not express Fc γ RIIA (Qiu et al., 1990; Hulett and Hogarth, 1994), the Fc γ RIIA transgenic mouse provides a useful tool for examination of Fc γ RIIA function (Taylor et al., 2000). When Fc γ RIIA TG mice and WT mice were injected via tail vein with ¹²⁵I-labeled human HA-IgG (200 μ l, 100 μ g/ml), the radioactivity associated with gel filtered platelets isolated from Fc γ RIIA TG mice was >5 fold greater than that from platelets isolated from WT mice (Figure 2). These data further illustrate that association of platelets with HA-IgG can occur *in vivo* and that it is dependent on the expression of Fc γ RIIA.

Internalization of IgG coated particles by platelets

We next turned to examine mechanisms by which platelets could participate in immune complex clearance. In phagocytes, small immune complexes are internalized through the process of endocytosis (actin independent), and larger particulate immune complexes are internalized through the process of phagocytosis (actin dependant). We previously demonstrated that human platelets via Fc γ RIIA can endocytose small IgG complexes such as heat-aggregated IgG (Worth et al., 2006). Since platelets *in vivo* may be exposed to IgG immune complexes in the macromolecular size range (e.g. microorganisms in host defense and nucleoprotein complexes in autoimmune disorders), we examined platelet ingestion of IgG coated beads sized from 0.1 to ~ 1.0 μ m (Figure 3). We observed that human platelets can readily internalize both small and large IgG-coated beads. After incubation at 37 °C for 20 min, platelet internalization of IgG coated 0.13 micron beads was 87%, and internalization of the IgG coated 0.97 micron beads was 65%.

In phagocytes, IgG-coated beads of 1.0 μ m and larger trigger the actin cytoskeleton rearrangement required for phagocytosis (Koval et al., 1998) while smaller IgG-coated beads do not trigger actin cytoskeleton rearrangement, or minimally so. The internalization of IgG coated beads by human platelets follows a similar pattern. In the presence of cytochalasin D (10 μ M), an inhibitor of actin rearrangement and phagocytosis (Casella et al., 1981, May and Machesky, 2001), the internalization of IgG coated 0.97micron beads by human platelets was almost completely abolished, while the ingestion of IgG coated 0.13 micron beads was very minimally affected (Figure 3). Thus, as in professional phagocytes, the internalization of large IgG complexes by human platelets is dependant on actin

rearrangement. However, unlike true phagocytes, neither killing nor digestion of microorganisms within platelets has been demonstrated (White, 2006). Nevertheless, the ability of platelets to engulf large IgG coated particulates may provide another mechanism for sequestering bacteria for clearance.

Platelet satellitism

We also investigated the interaction of IgG coated platelets with phagocytes. In examining freshly isolated monocytes from healthy individuals by light microscopy, we noted that many of the monocytes were ringed by small particles whose size appeared consistent with that of platelets. To determine whether these particles were indeed monocyte-bound platelets, we exposed monocytes from normal donors to a monoclonal antibody directed against CD61, a platelet specific surface marker, and, using flow cytometry, analyzed for monocyte-associated CD61 in the cell population gated for monocytes. The large increase in fluorescence intensity for anti-CD61 antibody-treated cells compared to the isotype control and untreated cells (Figure 4) identifies the particles bound to the monocytes *in vivo* as platelets.

The rosetting/binding of platelets around leukocytes is termed platelet “satellitism” (Criswell et al., 2001). Platelet satellitism is considered an uncommon phenomenon and has been most often reported for neutrophils in blood that has been anti-coagulated with EDTA (Criswell et al., 2001; Bizzaro, 1991; Morselli et al, 1999). Some studies have suggested that it occurs more frequently in disease states, especially conditions involving thrombotic complications, but others have found no correlation between the presence of disease and platelet satellitism (Criswell et al., 2001). Our results indicate that platelet satellitism also occurs on monocytes isolated from heparinized blood of healthy individuals (Figure 4).

Phagocytosis of platelets by circulating leukocytes

The coating of platelets with IgG complexes renders the platelets susceptible to destruction by phagocytes. The ability of monocytes and neutrophils to phagocytose HA-IgG platelet complexes *in vitro* is illustrated in Figure 5A. In this experiment, monocytes or neutrophils were bound at 0°C to platelets that had been coated *in vitro* with HA-IgG. The temperature of one aliquot was then raised to 37°C, the temperature conducive to internalization of IgG complexes, while the other remained at 0°C, as a binding control. The remaining external platelets were then labeled with PE-conjugated F(ab')₂ goat anti-human IgG (see Methods). The cell surface fluorescence of monocytes and neutrophils in cells incubated with platelets at 37°C was greatly reduced relative to cells maintained at 0°C (Figure 5A), suggesting that internalization (phagocytosis) of the HA-IgG coated platelets had occurred.

To determine whether the decrease of HA-IgG platelet complexes on the surface of the phagocytes after incubation at 37°C is due to their internalization or to dissociation from the cell surface, we also examined the fluorescence of monocytes that had been incubated with platelets bound to FITC-labeled human HA-IgG (Figure 5B). The FITC-labeled human HA-IgG allows the detection of both internalized and external platelets on monocytes. Thus, the similarity of fluorescence associated with the monocytes at 37°C and 0°C indicates the maintenance of FITC-HA-IgG levels and strongly suggests that the loss of PE staining from the cell surface shown in Figure 5A is due to the internalization of the HA-IgG platelet complexes (Figure 5B). Similar results were observed for neutrophils (not shown).

The electron micrographs of Figure 6 demonstrate that monocytes from healthy individuals also bind and internalize human platelets *in vivo*. The phagocytosis of platelets by leukocytes *in vivo* has been generally associated with disease states (Gasparyan et al., 2010; Lee and Tripathy, 2000; Maugeri et al., 2009). For example, in ITP, anti-platelet IgG

antibodies bound to the surface of platelets have been shown to promote the premature destruction of platelets by splenic macrophages (Gasparyan et al., 2010). Further, the internalization of platelets seen in freshly isolated leukocytes has been considered an *in vitro* phenomenon, related to the effect of anticoagulant EDTA on hidden determinants of platelet membrane glycoproteins (Bizzaro, 1991; White et al., 1978). Our micrographs demonstrate platelet internalization by monocytes from healthy individuals whose blood was anti-coagulated with heparin. Our data also illustrate that although splenic macrophages are considered the major site for destruction of IgG complexes and IgG-coated cells, including IgG-coated platelets, platelets are also internalized by circulating phagocytes *in vivo*.

EDTA dependant platelet satellitism has been shown to be antibody-mediated (Pegels et al., 1982; von dem Borne et al., 1986) and in some cases platelet satellitism has been associated with the ability of leukocytes to phagocytose platelets (Bizzaro, 1991; White et al., 1978). The occurrence of platelet satellitism in our preparations of monocytes (Figure 4), and the observations that platelets from healthy individuals bind IgG complexes *in vivo* (Figure 1A), suggest that the platelets bound and internalized by the monocytes shown in Figure 6B-6D are platelets to which IgG complexes have been bound *in vivo*.

We have provided further evidence that human platelets can internalize both small and large IgG coated particles. We have also provided evidence that Fc γ RIIA, by facilitating the binding of IgG complexes by platelets, provides a mechanism whereby IgG complexes can be sequestered for delivery to and destruction by circulating phagocytes. We propose that because of their large numbers, platelets provide a powerful system for clearance of IgG complexes both in disease states and in healthy individuals.

Acknowledgments

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Abbreviations

HA-IgG	Heat aggregated IgG
Mono-IgG	monomeric IgG
MF	mean fluorescence
TG	transgenic
WT	wild type

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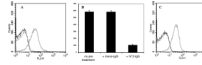


Figure 1.

(A) Human platelets bind IgG *in vivo*. Gel-filtered human platelets were incubated with PE-labelled F(ab')₂ goat anti-human IgG-PE (grey line), PE-labelled F(ab')₂ goat anti-rabbit IgG (dashed line), or only saline (Solid black line). Binding of IgG complexes on the surface of platelets was detected by flow cytometry.

(B) Human platelets can bind heat aggregated (HA-IgG) in the presence of physiologic concentrations of monomeric IgG. Human platelets were incubated with monomeric IgG (10 mg/ml), mAb IV.3 (25 µg/ml) or saline before exposure to HA human IgG. IgG bound to the platelet surface was detected by flow cytometry using PE-tagged F(ab')₂ goat anti-human IgG.

(C). FcγRIIA binds aggregated HA-IgG but not monomeric IgG. COS cells transfected to stably express FcγRIIA were incubated with monomeric human IgG (dashed line), HA-IgG (grey line) or saline (solid black line). IgG bound to the platelet surface was detected by flow cytometry using PE-tagged F(ab')₂ goat anti-human IgG. Flow cytometry using PE-tagged F(ab')₂ goat anti-human IgG detected only aggregated IgG on the cell surface.

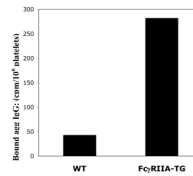


Figure 2.

Comparison of HA human IgG binding *in vivo*: Platelets from FcγRIIA TG mice vs wild type (WT) mice. ¹²⁵I-labeled HA human IgG was injected via tail vein into WT and FcγRIIA TG mice. After 5 min, the animals were sacrificed and platelets isolated. The radioactivity associated with platelets was determined in gel-filtered platelets. Wild type (43 cpm/10⁶ platelets); FcγRIIA TG mice (282 cpm/10⁶ platelets).

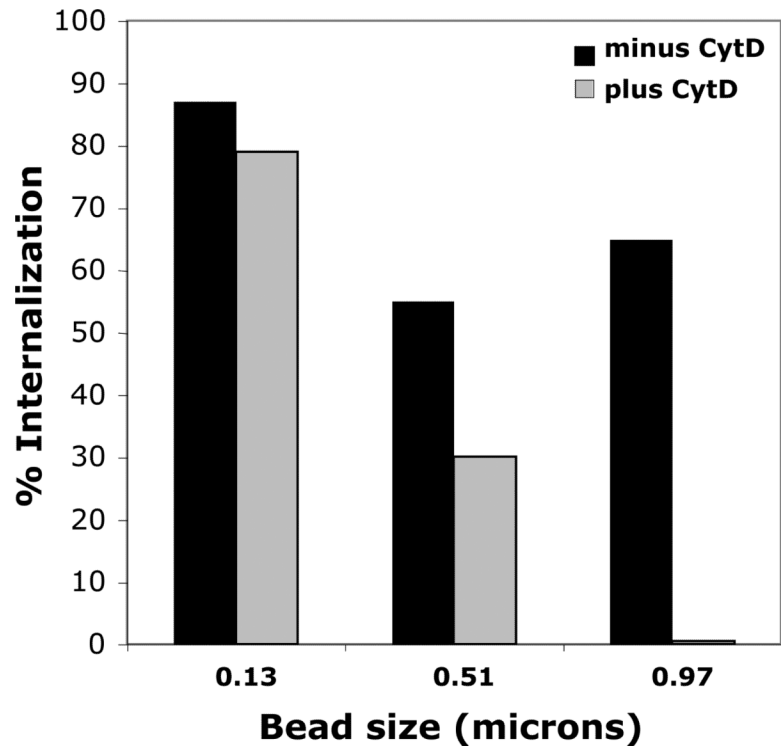


Figure 3. Platelets internalize both small and large IgG-coated beads. Streptavidin conjugated polystyrene beads coated with biotin/anti-biotin mAb were incubated with human platelets as described in Methods. Goat anti-mouse IgG conjugated to phycoerythrin (PE) was added to label remaining externally bound IC polystyrene beads and cell fluorescence was analyzed by flow cytometry.

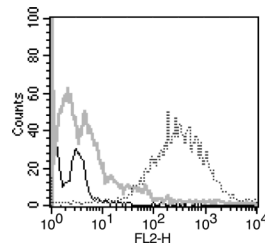


Figure 4. Detection of platelet-monocyte complexes by flow cytometry. Freshly isolated human monocytes were treated with antibody targeted to CD61, a specific platelet surface marker (dotted line), isotype control IgG (grey line) or saline (solid line). The secondary antibody was PE-labeled goat anti-mouse IgG. The platelet surface marker CD61 was detected on the cell population gated for monocytes.

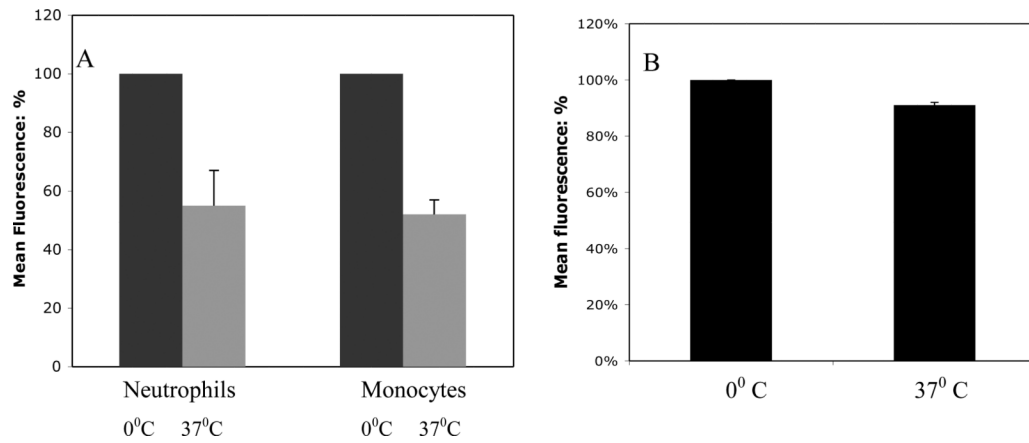


Figure 5.

Determination of phagocytosis of HA-IgG platelet complexes by leukocytes *in vitro*. A. Gel filtered human platelets were treated with HA-IgG before incubation at 0°C or 37°C with freshly isolated monocytes or neutrophils (see Methods). PE-labelled goat anti-human IgG was used to label HA-IgG platelet complexes remaining on the surface of the leukocytes. Fluorescence was analyzed in cell populations gated for monocytes or neutrophils. The loss in surface bound HA-IgG platelet complexes at 37°C, as indicated by decreased mean fluorescence (MF) of the cells, suggests their internalization by the phagocytic cells. Using the formula $(MF\ 0^{\circ}C - MF\ 37^{\circ}C)/MF\ 0^{\circ}C \times 100$, we estimated the % phagocytosis by neutrophils as 42% and phagocytosis by monocytes as 45%.

B. Monocytes were incubated with FITC-labeled HA-IgG platelet complexes in order to examine total cell associated labeling (i.e. both external and internalized HA-IgG platelet complexes) at 0°C and 37°C. The similarity of fluorescence at 37°C and 0°C suggests that the FITC-IgG platelet complexes are internalized by the monocytes and not shed from the surface.

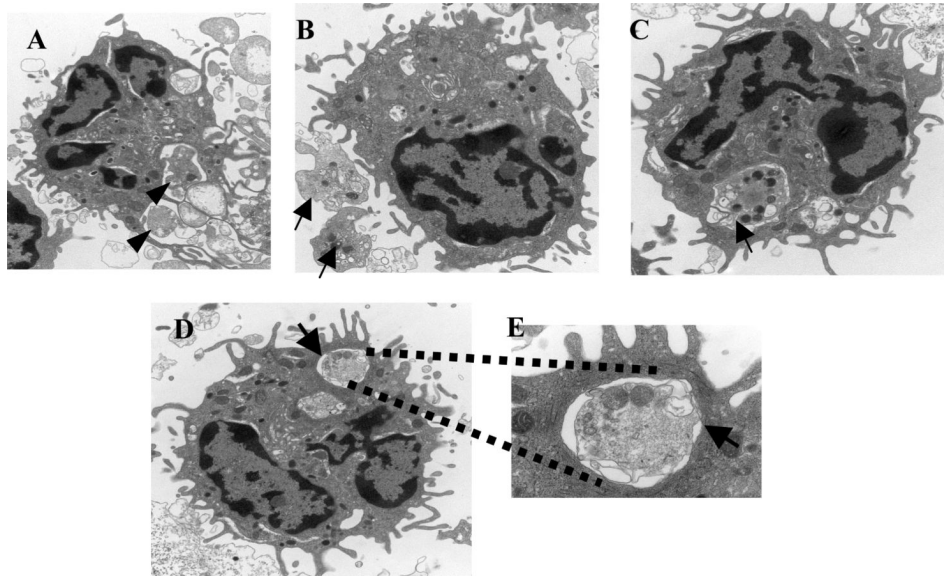


Figure 6. Representative electron micrographs demonstrating the binding and phagocytosis of platelets by monocytes from the same normal donor. Arrows indicate location of platelets. A and B. Binding of platelets to human monocytes. C and D. Phagocytosis of platelets by monocytes. E. Enlarged image of internalized human platelet from panel D. In B, C and D, *in vivo* binding and phagocytosis of platelets by monocytes is demonstrated using freshly isolated monocytes. In A, monocytes were incubated with platelets pre-coated with HA-IgG before processing for EM. Human monocytes and platelets were prepared from heparinized venous blood.