Enhancement of Platelet Reactivity and Modulation of Eicosanoid Production by Intact Erythrocytes

A New Approach to Platelet Activation and Recruitment

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Abstract

Erythrocytes are known to influence hemostasis. Bleeding times are prolonged in anemia and corrected by normalizing the hematocrit. We now demonstrate that intact erythrocytes modulate biochemical and functional responsiveness of activated platelets. A two-stage procedure, permitting studies of cell–cell interactions and independently evaluating platelet activation and recruitment within 1 min of stimulation, was developed. Erythrocytes increased platelet serotonin release despite aspirin treatment, enzymatic adenosine diphosphate removal, protease inhibition, or combinations thereof. The data suggested that erythrocyte enhancement of platelet reactivity can reduce the therapeutic effectiveness of aspirin.

Erythrocytes metabolically modified platelet arachidonate or eicosapentaenoate release and eicosanoid formation. They promoted significant increases in cyclooxygenase and lipoxygenase metabolites upon platelet stimulation with collagen or thrombin. However, with ionophore, erythrocytes strongly reduced platelet lipoxygenation. These erythrocyte modulatory effects were stimulus-specific. Activated platelet-erythrocyte mixtures, with or without aspirin, promoted 3–10-fold increases in extracellular free fatty acid, which would be available for transcellular metabolism. Erythrocyte-induced increases in free eicosapentaenoate may contribute to antithrombotic and anti-inflammatory effects of this fish oil derivative.

These results provide biochemical insight into erythrocyte contributions to thrombosis and hemostasis, and support the concept of thrombus formation as a multicellular event. (*J. Clin. Invest.* 1991. 87:571–580.) Key words: arachidonic acid • aspirin • cell–cell interactions • eicosapentaenoic acid • serotonin release

Introduction

An important consequence of platelet activation is release of intracellular components that in turn activate additional platelets and interact with other cells in the microenvironment, i.e., the "recruitment phase" of hemostasis or thrombosis (1). We devised an experimental system to study cell–cell interactions and independently evaluate platelet activation and platelet recruitment (2, 3) as separate components of platelet reactivity. Platelets, combined suspensions of platelets with other cells, or even whole blood are stimulated (generating system), immediately centrifuged and the separated cell-free releasate studied biochemically. In addition, the releasate can be added to human platelet-rich plasma (PRP)[1] (assay system) where it acts as agonist for initiation and evaluation of recruitment (Fig. 1).

Previous concepts of erythrocyte participation in hemostatic and thrombotic events focused on mechanical liberation of proaggregatory substances (4). In contrast, we found that metabolically active, intact erythrocytes enhanced platelet activation and recruitment.

Eicosanoid metabolism is modulated by cell–cell interactions wherein precursors, intermediates and metabolic end products can be processed by different cells in close apposition and under different stimulatory conditions (5). We therefore examined metabolism of platelet arachidonate and eicosapentaenoate (a biologically active ω-3 fatty acid derived from fish oil) when platelets were activated alone or in combination with erythrocytes in the presence or absence of aspirin. Erythrocytes considerably modified platelet eicosanoid metabolism and functional responsiveness. The rapid cell–cell interactions described herein have not been heretofore characterized.

Methods

Platelet and erythrocyte collection and processing. Cell suspensions were prepared from 160 ml of blood, collected by free flow in plastic tubing through a 15-gauge needle (6). 50-ml polypropylene tubes were used, two of which contained 4.5 ml of 3.8% sodium citrate in a total volume of 45 ml. Two others contained 6 ml of acid-citrate-dextrose (ACD; 38 mM citric acid, 75 mM sodium citrate, and 135 mM glucose) in a total volume of 46 ml. Blood was centrifuged at 200 *g* to obtain PRP. The PRP, prepared with citrate (adjusted to a platelet count of $4–5 \times 10^8$/ml with platelet-poor plasma) was capped and maintained at 22°C under 5% CO_2-air, for use as the assay system for platelet aggregation (6). Platelets in the ACD-anticoagulated tubes were washed and labeled as described below. Washed platelets were used in the generating system to be stimulated by agonists in the presence and absence of washed erythrocytes.

After removal of PRP from the citrate-anticoagulated tubes, the buffy coat was aspirated and discarded. 10 ml of erythrocytes were

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1. *Abbreviations used in this paper:* ACD, acid citrate dextrose (USP Formula A); ASA, acetylsalicylic acid; CP, creatine phosphate; CPK, creatine phosphokinase; EPA, eicosapentaenoic acid; 12-HHTE, 12(S)-hydroxyheptadecatetraenoic acid; 12-HHTrE, 12(S)-hydroxyheptadecatrienoic acid; 12-HEPE, 12(S)-hydroxy-5,8-*cis*,10-*trans*,14,17-*cis*-eicosapentaenoic acid; 12-HETE, 12(S)-hydroxy-5,8-*cis*,10-*trans*,14-*cis*-eicosatetraenoic acid; 5-HT, serotonin; LDH, lactic dehydrogenase; PRP, platelet-rich plasma.

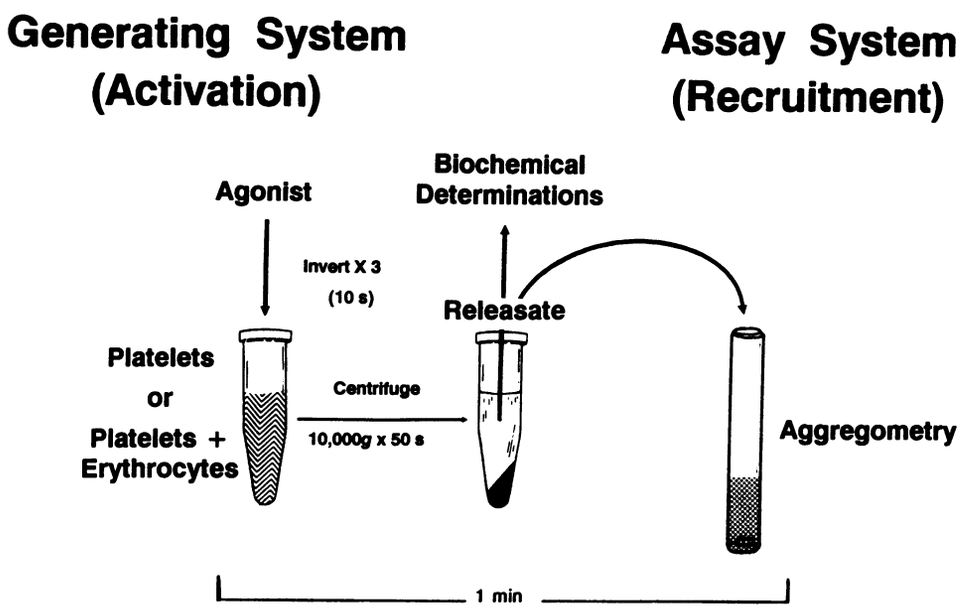

Figure 1. Diagram of systems for evaluation of platelet activation (generating system) and recruitment (assay system).

removed from the central area of the erythrocyte zone with a plastic pipette. Erythrocytes were distributed in 3-ml aliquots and 7 ml of phosphate-buffered saline, containing glucose (NaCl 130.9 mM, Na_2HPO_4 5.1 mM, KH_2PO_4 1.50 mM, glucose 83 mM, pH 7.2) were added to each aliquot. After gentle inversion, the erythrocyte suspension was centrifuged at 200 *g* (10 min, 22°C) and the procedure repeated. At each washing step, the top 2 mm of erythrocytes were discarded. Erythrocytes were stored at 4°C until use.

Platelet labeling procedures for arachidonate, eicosapentaenoate, and serotonin. The ACD-anticoagulated PRP was centrifuged at 90 *g* for 10 min (22°C) to eliminate any residual erythrocytes. Erythrocyte-free PRP was acidified to pH 6.5 with 0.1 volume citrate solution (38 mM citric acid, 75 mM sodium citrate) and centrifuged at 2,000 *g* (15 min at 22°C). The resulting pellet was gently resuspended with a plastic transfer pipette in 2 ml Tris-citrate buffer (63 mM Tris, 95 mM NaCl, 5 mM KCl, 12 mM citric acid, 5.5 mM glucose, and 0.01% bovine serum albumin, pH 6.4), transferred to another calibrated polypropylene tube, and the volume adjusted to 3 ml. For labeling, the suspension was transferred to a 50-ml plastic container and 33 ml of Tris-citrate buffer added. 250 μCi [^{3}H] arachidonic acid, 191 Ci/mmol, or 250 μCi [^{3}H] eicosapentaenoic acid (EPA), 79 Ci/mmol (Du Pont NEN Research Products, Boston, MA) were converted to the sodium salt with Na_2CO_3 and transferred to the platelet suspension (6). Labeling was carried out for 45 min with gentle shaking (37°C). The sample was cooled on ice (10 min), transferred to calibrated conical tubes, and centrifuged at 1,450 *g* for 15 min (4°C). The washing procedure was repeated once. Final suspension was in cold 0.9% NaCl. The platelet count was adjusted to 10^9/ml. Scintillation counting was performed on a 20-μl aliquot from the adjusted platelet suspension. Uptake of label averaged 57% for arachidonate and 48% for eicosapentaenoate.

Radiolabeled serotonin ([^{14}C] 5-HT) creatinine sulfate, 54 mCi/mmol (in 2% ethanol) was obtained from Amersham Corp., Arlington Heights, IL. To label platelets, 0.2 nmol [^{14}C] 5-HT was added to the anticoagulant for each milliliter anticoagulated whole blood. 1 h after blood collection, scintillation counting of 50 μl of PRP was carried out for measurement of uptake as compared to 50 μl of platelet-poor plasma. Uptake of [^{14}C] 5-HT averaged 96%. The washing procedure was then continued. Imipramine (2.5 μM final concentration) was added 1 min before agonist to prevent re-uptake of released 5-HT.

In radiolabeling experiments, activation was quantified as total released radioactivity by counting 50 μl cell-free releasate (see below) in 4 ml Aquasol-2 (Du Pont NEN Research Products).

System for independent evaluation of platelet activation and recruitment. Fig. 1 depicts the two-stage in vitro system used. Agonists were added to the generating system to activate platelets or combined suspensions of platelets and erythrocytes. The cell-free releasate obtained after centrifugation of the generating system was, within 1 min, either transferred to the assay system (PRP) for assessment of proaggregatory activity (recruitment) or used for biochemical studies to assess activation.

The generating system (1 ml in total volume) consisted of 0.2 ml of washed platelets containing 1×10^9/ml, 0.4 ml of phosphate-buffered saline, and 0.4 ml of packed erythrocytes or buffer controls. This was preincubated for 10 min at 37°C before agonist addition. After addition of agonist, the tube was inverted three times (10 s), followed by centrifugation (Eppendorf, Brinkmann Instruments Co., Westbury, NY) at 10,000 *g* for 50 s. The cell-free supernatant (releasate) was immediately transferred (50 μl) to the PRP assay system in an aggregometer, to serve as agonist for platelet aggregation (recruitment). The assay system consisted of 125 μl of PRP containing $4–5 \times 10^8$ platelets/ml, 100 μl of phosphate-buffered saline and calcium (final concentration 1 mM). With collagen, there was no carryover of agonist from the generating system since it sedimented during centrifugation. Carryover of thrombin and ionophore had no appreciable effect in the assay system (see Fig. 3). Buffer blanks or ethanol controls were inert.

Human thrombin (provided by Dr. John Fenton II) was used at 1 U/ml, collagen at 4 or 16 μg/ml (Hormon-Chemie, Munich, FRG), and ionophore A23187 (Sigma Chemical Co., St. Louis, MO) at 2 μM final concentration. The latter was added in 1 μl of ethanol. With thrombin and collagen, the generating system contained 1 mM calcium. With ionophore, no calcium was added. Where used, aspirin (in saline, 1 mM final concentration) was added before preincubation. For enzymatic removal of released ADP from the generating system, 2 U/ml apyrase (Sigma Chemical Co. grade I) or 5 mM creatine phosphate (CP) plus 40 U/ml creatine phosphokinase (CPK; Sigma Chemical Co.) were added during preincubation, 1 min before stimulation. A protease inhibitor mixture (phenylmethylsulfonyl fluoride [PMSF] 1 mM, pepstatin A 1 mM, leupeptin 100 μM, 2.0% ethanol) was added to the generating system 1 min before agonist in specified experiments. Platelets and erythrocytes were studied in the physiological range, i.e., 2×10^8 platelets/ml and erythrocytes at 40% hematocrit.

Lipid analyses. Cell-free releasate (400 μl) was brought to pH 3.5 with 1 M citric acid, 1.4 ml chloroform/methanol (2:5) added, and the polypropylene tube vortexed. Samples could be stored at −70°C, before modified Bligh and Dyer extraction (6). Final extracts were dried

under nitrogen, dissolved in 0.1 ml chloroform/methanol (2:1) and aliquots removed for scintillation counting. Thin-layer chromatography, scanning, and quantitation have been described (6).

Procedures for evaluation of cell integrity. Two methods were employed to verify that results were not due to loss of cell integrity. Washed platelets alone with collagen (16 μg/ml), thrombin or vehicle all showed 0.2% lactate dehydrogenase (LDH) release (measured as described [7]). Ionophore induced 0.79% LDH release and the ethanol vehicle 0.33%. Platelet-erythrocyte mixtures in buffer showed 0.07% LDH in the supernatants, and this remained constant with collagen and thrombin stimulation. With ionophore, 0.11% LDH was noted.

Hemoglobin content of supernatants from samples containing erythrocytes was measured spectrophotometrically at 576 and 540 nm. Supernatants of erythrocytes alone in buffer contained 0.05% hemoglobin. With 0.1% ethanol, 0.23% hemoglobin was present. Aspirin had no effect on supernatant hemoglobin or LDH values. In no instance did platelet activation induce hemoglobin or LDH release from erythrocytes.

Results

Effect of intact erythrocytes on platelet activation and recruitment. Stimulation of platelets in the presence of intact erythrocytes in the generating system (Fig. 1) resulted in marked enhancement of platelet activation, as indicated by 5-HT release (Fig. 2). Erythrocyte amplification of platelet reactivity was also reflected in the ability of the cell-free releasate, when used as agonist, to induce an increased aggregation response in the PRP assay system (recruitment) (Fig. 3).

Addition of collagen (16 μg/ml) to platelets alone evoked 34% 5-HT release (Fig. 2). This rose to 81% release in the presence of erythrocytes. Aspirin reduced the collagen response to 15%, but with added erythrocytes release increased to 56%.

The amplifying effect of erythrocytes was lower with the soluble agonists, thrombin and especially ionophore, as compared to collagen (Fig. 2). Erythrocyte enhancement (expressed as percentage of control) was similar, even with lower concentrations of thrombin (0.1, 0.3 U/ml) or ionophore (0.5 μM).

Releasates from platelets alone and from platelet-erythrocyte suspensions were also tested in the PRP assay system for recruitment (Fig. 3). When collagen (4 μg/ml) was added to platelets alone in the generating system, an aliquot of releasate, transferred to the PRP assay system, induced aggregation with a maximal amplitude of 26±7 mm (mean±SEM). However, releasates from platelets plus erythrocytes, resulted in enhanced recruitment, with aggregation curves of 62±9 mm ($P < 0.01$). The erythrocyte-amplifying effect on platelet recruitment with 16 μg/ml collagen (Fig. 4) was less than that at 4 μg/ml.

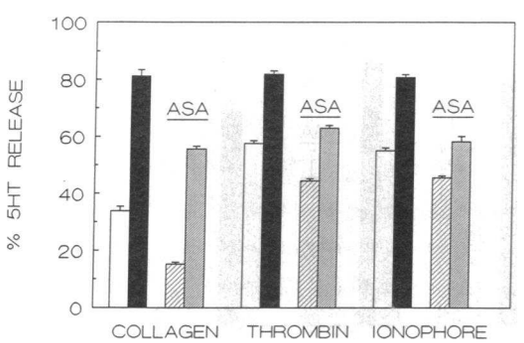

Figure 2. Serotonin release by platelets in the presence or absence of erythrocytes, with and without ASA. Collagen 16 μg/ml; thrombin 1 U/ml; ionophore A23187 2 μM. $P < 0.001$ for platelets vs. platelet-erythrocyte mixtures; however, $P < 0.01$ in ASA-treated ionophore-stimulated samples. Results represent five separate experiments, done in triplicate. (□) Untreated platelets alone; (■) untreated platelets + erythrocytes; (▨) ASA-treated platelets alone; (▩) ASA-treated platelets + erythrocytes.

Control experiments defined absolute requirements for releasates from the generating system to induce recruitment in the PRP assay system (Fig. 3). Erythrocytes must interact with activated platelets for enhancement to occur. Supernatants from unstimulated platelet–erythrocyte mixtures, or from erythrocytes alone exposed to agonists did not induce recruitment, or 5-HT release. In additional control experiments, freshly prepared, washed erythrocytes were compared with glutaraldehyde-treated, ATP-depleted erythrocytes (8). In contrast to fresh erythrocytes, glutaraldehyde-treated erythrocytes had no enhancing effect on platelet activation or recruitment. Thus, metabolic integrity of erythrocytes is required for the cell interactions under study.

Releasates from platelets stimulated by thrombin or ionophore in most instances induced reversible aggregation. Inclusion of intact erythrocytes markedly increased recruiting properties of releasates, resulting in irreversible aggregation (Fig. 3).

Aspirin (ASA)-treated platelets responded to collagen (16 μg/ml) or thrombin with releasates inducing aggregation of 51±19 or 40±9 mm amplitude, respectively (Fig. 4). Releasates from ASA–platelet–erythrocyte mixtures extended the amplitudes to 76±15 mm (collagen), and 59±10 mm (thrombin). With ionophore stimulation of ASA-treated platelets recruitment decreased to a greater extent than with collagen or thrombin. In this setting, ionophore-induced responses were still amplified by erythrocytes (Fig. 4). Activation did not parallel recruitment with ionophore stimulation of ASA-treated cells (compare Figs. 2 and 4).

Effects of enzymatic removal of ADP in the generating system on platelet–erythrocyte interactions. Since erythrocyte-derived ADP has been implicated as an agonist for platelet function (9), we studied the generating system in its absence. Serotonin release from collagen-stimulated platelets was reduced by apyrase or CP/CPK (Fig. 5). Nevertheless, ADP removal did not affect erythrocyte enhancement of collagen-stimulated 5-HT release. ADP removal from thrombin- and ionophore-stimulated platelets alone or platelet–erythrocyte mixtures did not affect 5-HT release. Erythrocyte enhancement took place with all agonists, and was most pronounced with collagen. Therefore enhancement of platelet activation by erythrocytes was not primarily mediated by ADP, nor was released platelet ADP required to elicit the erythrocyte effect (Fig. 5). The effects of ADP removal on platelet recruitment (Table I) were much more pronounced than those on platelet activation, especially with collagen.

ASA treatment was superimposed upon removal of ADP from the generating system to establish (*a*) whether erythrocytes still reacted to material released from platelets in the absence of both TXA_2 and ADP, and (*b*) whether these platelets still responded to erythrocytes. In the collagen-stimulated, ASA-treated, ADP-free generating system, 5-HT release in platelets alone was markedly reduced, but erythrocyte enhancement still occurred (Fig. 5). With thrombin and ionophore,

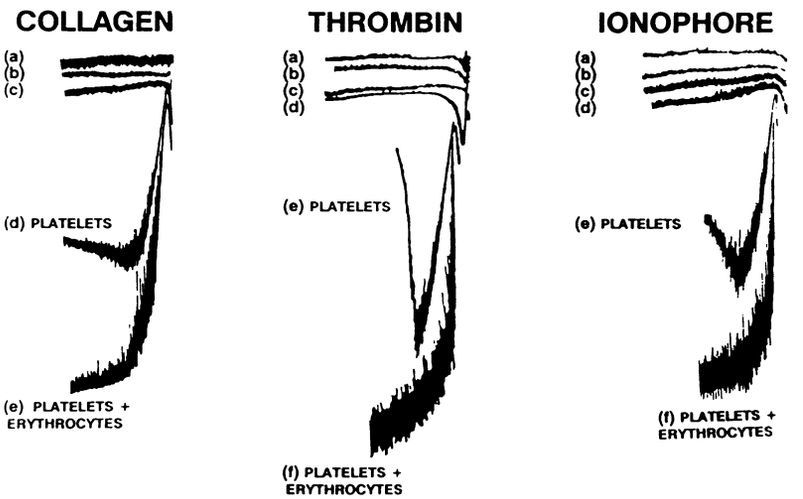

Figure 3. Aggregation responses in PRP (recruitment) induced by cell-free releasates from washed platelets or platelets plus erythrocytes, activated by collagen, thrombin or ionophore. *Left panel:* (*a*) Washed platelets, buffer. (*b*) Washed platelets, erythrocytes, buffer. (*c*) Erythrocytes, collagen, no platelets. (*d*) Releasate from collagen-activated platelets added to PRP assay system. (*e*) Releasate from collagen-activated platelets plus erythrocytes added to PRP assay system. Collagen 4 μg/ml. *Middle panel:* (*a*) Washed platelet-buffer control. (*b*) Washed platelets, erythrocytes, buffer. (*c*) Erythrocytes, thrombin, no platelets. (*d*) Thrombin, buffer, no platelets or erythrocytes. (*e*) Releasate from thrombin-activated platelets added to PRP assay system. (*f*) Releasate from thrombin-activated, platelet-erythrocyte mixture. Thrombin 1 U/ml. *Right panel:* (*a*) Washed platelets, ethanol (vehicle) control. (*b*) Washed platelets, erythrocytes, ethanol control. (*c*) Ionophore, erythrocytes. (*d*) Ionophore, buffer. (*e*) Releasate from platelets, stimulated by ionophore. (*f*) Releasate from ionophore-stimulated platelets plus erythrocytes. Ionophore 2 μM. Curves represent 18 separate experiments.

5-HT release from platelets alone was reduced by aspirin (20% as compared to 50% for collagen). This was unaltered by ADP removal. In all instances, erythrocytes enhanced 5-HT release with thrombin and ionophore to a lower degree than with collagen (Fig. 5). Thus, erythrocyte enhancement of platelet activation occurred with all three agonists, despite ADP removal and aspirin treatment.

Effects of protease inhibitors on platelet activation. When a protease inhibitor mixture was added to platelets alone, with or without ASA plus apyrase, there was no inhibition of 5-HT release with collagen or ionophore. However, in collagen-stimulated platelet–erythrocyte suspensions, protease inhibitors reduced but did not abolish erythrocyte enhancement of 5-HT release (Table II). This protease inhibitor effect occurred with collagen in untreated platelet–erythrocyte mixtures (18% reduction), as well as those pretreated with aspirin plus apyrase (33% reduction) when compared with vehicle controls (Table II). These data suggest participation of protease activity in platelet–erythrocyte interactions in samples stimulated with collagen ($P < 0.005$), but not ionophore. Since the PMSF component of the protease inhibitor mixture would directly affect thrombin by sulfonylating the active site serine, parallel experiments with thrombin were not performed.

Eicosanoid metabolism during platelet-erythrocyte interactions. When [^{3}H]arachidonate-labeled platelets were activated with collagen and thrombin, erythrocytes strongly amplified arachidonate release (Table III). Amplification of release was more pronounced with collagen than thrombin. Platelet release of [^{3}H] arachidonate by ionophore exceeded that of collagen and thrombin fivefold (Table III). In contrast to collagen and thrombin, ionophore-induced arachidonate release was unchanged by erythrocytes.

ASA pretreatment reduced arachidonate release induced by collagen (10, 11), thrombin, and especially ionophore (Table III). The aspirin-induced reduction of arachidonate release in platelets alone was overcome by erythrocytes with each agonist (Table III). Notably, erythrocytes promoted the same arachidonate release as platelets alone in the absence of aspirin.

Patterns of arachidonate transformation in platelets alone. At 1 min cyclooxygenase products (TXB_2, 12-HHTrE; see footnote 1) predominated with collagen and thrombin, but with ionophore a greater proportion of 12-HETE (see footnote 1) was produced (*open bars,* Fig. 6). With ASA-treated platelets alone, production of 12-HETE and free arachidonate were reduced at 1 min (compare Table IV and Fig. 6), reflecting the decrease in total radioactivity released in the presence of aspirin (Table III).

Erythrocyte modification of platelet eicosanoid production. Erythrocytes exerted stimulus-specific alterations in eicosanoid profiles of cell-free releasates. However, erythrocytes induced a marked increase in free arachidonate with all three agonists (Fig. 6, Table IV).

Erythrocyte amplification of platelet TXB_2 and 12-HHTrE was very prominent with collagen, modest with thrombin and

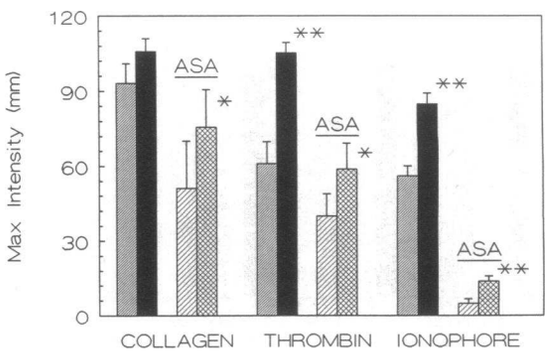

Figure 4. Effect of ASA on recruiting activity (aggregation response in PRP) of cell-free releasates from platelets or platelets plus erythrocytes in the generating system (Fig. 1). Collagen 16 μg/ml, thrombin 1 U/ml or ionophore 2 μM; mean±SEM; $n = 5$–11. *$P < 0.025$; **$P < 0.005$, platelets vs. platelet-erythrocyte mixtures, untreated or ASA-treated. (▨) Platelets; (■) platelets plus erythrocytes; (▨) aspirin-treated platelets; (■) aspirin-treated platelets plus erythrocytes.

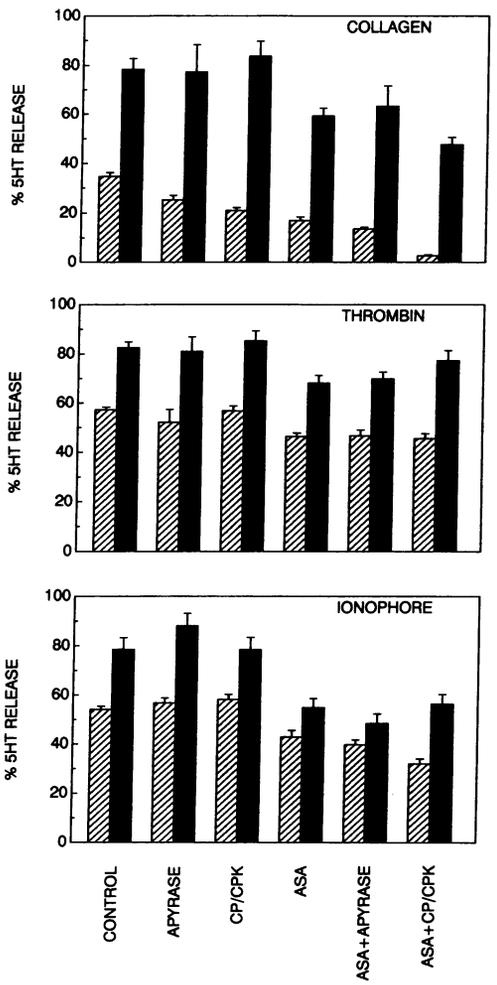

Figure 5. Effects of enzymatic removal of ADP in the presence or absence of aspirin on platelet-erythrocyte interactions. Serotonin release (activation) was measured 1 min after stimulation. Intact erythrocytes (solid bars) amplified platelet activation (hatched bars). Mean±SEM, $n = 5$; collagen 16 μg/ml, $P < 0.005$ for platelets vs. platelet-erythrocyte mixtures (paired t test); thrombin 1 U/ml, $P < 0.01$; ionophore 2 μM, $P < 0.05$; apyrase 2 U/ml; CP 5 mM; CPK 40 U/ml; aspirin 1 mM.

did not occur at all with ionophore (Fig. 6). The increment of platelet 12-HETE production by erythrocytes was 1.6-fold for collagen and 1.2-fold for thrombin. However, with ionophore erythrocytes actually inhibited 12-HETE production by one-half (Fig. 6).

As in the ASA-free system, the most prominent effect of erythrocytes in the presence of ASA was a marked increase in free arachidonate induced by all agonists (Table IV). Erythrocytes enhanced 12-HETE production with collagen and thrombin, but with ionophore a reduction was once again observed (Table IV).

These results demonstrate strong modulating effects of erythrocytes on platelet eicosanoid formation during the first minute of stimulation.

EPA metabolism during platelet-erythrocyte interactions. With EPA-labeled platelets alone, EPA release with collagen and thrombin was similar, but was about threefold greater with ionophore (Table V).

Release of EPA in the presence of erythrocytes was enhanced more with collagen than with thrombin as compared to platelets alone (Table V). Enhancement was similar to that with arachidonate-labeled platelets (Table III). Erythrocytes did not enhance ionophore-induced EPA release, again comparable to arachidonate (Tables III and V). Released radioactivity with ionophore in platelet–erythrocyte mixtures was lower with EPA than with arachidonate.

Aspirin reduced EPA release from stimulated platelets in the presence or absence of erythrocytes (Table V). Under these circumstances, erythrocytes also enhanced release of EPA with collagen and thrombin, as with arachidonate. However, with ionophore, the ASA-induced decrease in radioactivity in platelets alone was not reversed by erythrocytes, in contrast to results with arachidonate-labeled platelets (compare Tables III and V).

EPA released from platelets alone by each agonist was preferentially processed by lipoxygenase (Table VI). This differed from arachidonate with collagen and thrombin, where cyclooxygenase products predominated (Fig. 6). In the arachidonate-ASA experiments, 12-HETE and free arachidonate were reduced as compared to ASA-free controls (compare Table IV and Fig. 6). In contrast, in the EPA-ASA experiments, 12-HEPE (see footnote 1) and free EPA were essentially unaffected by ASA (compare Tables VI and VII).

Erythrocytes significantly altered the EPA-derived eicosanoid profiles with each agonist (Table VI). With ionophore, erythrocytes markedly decreased platelet 12-HEPE; with collagen, TXB_3 and 12-HHTE (see footnote 1) were enhanced. As with arachidonate, free platelet EPA was dramatically increased by platelet-erythrocyte contact with each agonist (Table VI).

Upon ASA treatment, erythrocytes inhibited platelet 12-HEPE production drastically with ionophore but not signifi-

Table I. Effects of ADP Removal on Platelet Activation and Recruitment

	Relative response*			
	Collagen		Thrombin	
	Apyrase‡	CP + CPK§	Apyrase	CP + CPK
	%			
Platelets alone				
Activation	73	61	91	99
Recruitment	0	0	36	45
Platelets + erythrocytes				
Activation	102	107	98	103
Recruitment	0	0	58	70

Agonists were added to [^{14}C]5HT-labeled platelets (2×10^8/ml) or to labeled platelets plus erythrocytes (40% hematocrit) in the absence (100% = control) or presence of ADP-removing enzymes. Activation was measured as 5-HT release; recruitment was measured as proaggregatory activity of cell-free releasates (Methods).

* Data are expressed as percentage of controls (100%) in the absence of ADP-removing enzymes for platelets alone, and platelets plus erythrocytes, respectively.

‡ Apyrase, 2 U/ml.

§ CP, 5 mM and CPK, 40 U/ml.

Table II. 5-HT Release in Stimulated Platelets and Platelet–Erythrocyte Mixtures in the Presence of Protease Inhibitors, with and without ASA plus Apyrase

Treatment*	Collagen		Ionophore	
	Platelets alone	Platelets + erythrocytes	Platelets alone	Platelets + erythrocytes
	%			
Vehicle	25.02±3.48	79.47±5.68	36.27±1.67	66.35±2.36
Inhibitor mixture	22.93±2.24	65.16±0.62‡	34.51±1.12	63.51±6.50
ASA + apyrase + vehicle	16.86±2.34	63.84±9.20	31.98±3.93	43.77±2.27
ASA + apyrase + inhibitor mixture	17.47±4.68	42.90±1.44§	38.37±3.86	45.24±10.58

Agonists were added to [^{14}C]5-HT-labeled platelets (2×10^8/ml) or to platelet–erythrocyte mixtures (40% hematocrit) in the generating system, in the presence or absence of protease inhibitors, with and without ASA plus apyrase. 5-HT release was monitored 1 min after stimulation. Values are mean±SEM of duplicate assays in two separate experiments. * Collagen (16 μg/ml); ionophore (2 μM); vehicle (2% ethanol); inhibitor mixture: PMSF (1 mM) + pepstatin A (1 mM) + leupeptin (100 μM); ASA (1 mM); apyrase (2 U/ml). ‡ $P < 0.005$; § $P < 0.025$ using paired t test (with vs. without protease inhibitors).

cantly with collagen and thrombin. Erythrocytes again promoted a substantial increase in free EPA with each agonist (Table VII).

Thus, erythrocytes induced distinct differences in eicosanoid metabolism when platelets were labeled with EPA or arachidonate, especially in the lipoxygenase pathway. Erythrocytes increased 12-HETE formation with and without ASA treatment with collagen and thrombin. In sharp contrast, erythrocytes did not increase 12-HEPE production from EPA in the same setting. Results with ionophore were unique: erythrocytes markedly reduced production of 12-HETE and especially 12-HEPE (compare Fig. 6 and Tables IV, VI, and VII). Erythrocyte effects on cyclooxygenation and promotion of free fatty acid accumulation were similarly pronounced in EPA- and arachidonate-labeled platelets.

Discussion

The system developed for independent evaluation of platelet activation and recruitment serves as an in vitro model of early hemostatic and thrombotic events. It focuses on early time points and allows for measurement of biochemical and physiological parameters associated with activation and cell contact (Fig. 1). Intact erythrocytes enhanced platelet reactivity, as evidenced by activation (5-HT release) in the generating system (Figs. 2 and 5), and platelet recruitment (aggregation) induced by releasates added to the PRP assay system (Figs. 3 and 4). Platelet activation and intact erythrocytes were required for induction of the enhancing effect (Fig. 3). Whereas erythrocyte-derived ADP may play a role in platelet function in specific situations, as previously described (9, 12), our data unequivocally demonstrated that erythrocyte enhancement of platelet reactivity proceeded in the complete absence of released ADP (Fig. 5).

Enhancement was greater with collagen than with thrombin or ionophore (Fig. 2). This may be relevant to the chronology of an accumulating platelet thrombus in vivo. Platelets are initially exposed to collagen at sites of injury. Therefore erythrocyte enhancement of platelet reactivity would be functionally important at this early phase in hemostasis. Thrombin, which forms subsequent to collagen contact, induced twice as much activation with platelets alone as did collagen (Fig. 2). However, erythrocyte enhancement of platelet activation was lower with thrombin than with collagen, but could also support thrombus formation.

Partial reversal of the ASA-induced platelet activation defect by erythrocytes. Erythrocyte enhancement of ASA-treated platelet activation occurred with all three agonists despite ab-

Table III. Release of Radioactivity from [^{3}H] Arachidonic Acid–labeled Platelets or Platelet–Erythrocyte Suspensions Stimulated with Collagen, Thrombin, or Ionophore

Stimulus*	No aspirin		Aspirin	
	Platelets alone	Platelets + erythrocytes	Platelets alone	Platelets + erythrocytes
	dpm			
Collagen	9,120±950	22,590±1,507§	4,475±343	8,803±1,095‡
Thrombin	7,939±1,524	12,956±2,142‡	4,333±1,280	8,285±597‡
Ionophore	46,875±5,069	47,122±4,345	18,043±2,806	43,043±3,336‡

Agonists were added to [^{3}H]arachidonate-labeled platelets (2×10^8/ml) or to platelet-erythrocyte mixtures (40% hematocrit) in the generating system. Values are mean±SEM of disintegrations per minute in 50 μl of releasate from activated cells. n = 7–10. * Collagen (16 μg/ml); thrombin (1 U/ml); ionophore (2 μM). ‡ $P < 0.005$; § $P < 0.001$ using paired t test (platelets vs. platelets plus erythrocytes).

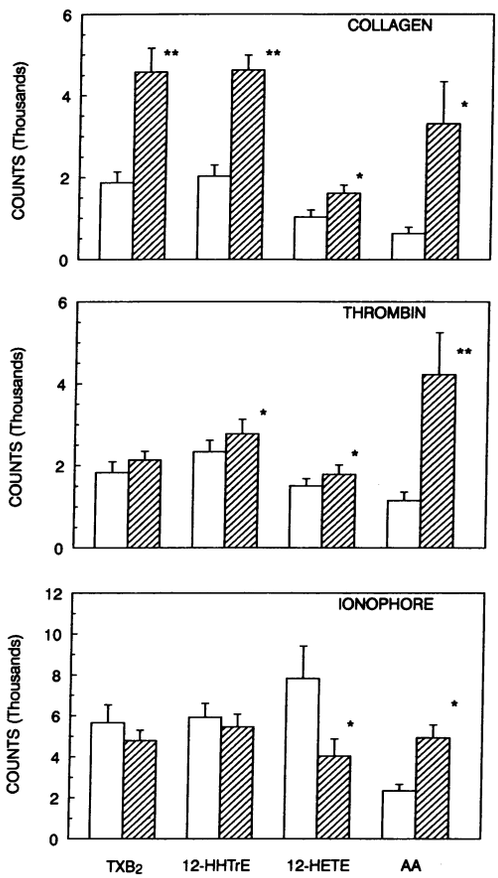

Figure 6. Effects of erythrocytes on eicosanoid profiles of releasates from [^{3}H]arachidonate-labeled platelets activated in the generating system. After stimulation in the presence (*hatched bars*) or absence (*open bars*) of erythrocytes, eicosanoids were isolated, separated and quantified by thin-layer chromatography and scanning. Mean±SEM; n = 7–10. *$P < 0.01$; **$P < 0.005$; collagen 16 μg/ml; thrombin 1 U/ml; ionophore 2 μM. Note different ionophore scale.

sence of TXA_2 and HHTrE. In contrast to thrombin and ionophore, activation by collagen was more vulnerable to cyclooxygenase inhibition, but more responsive to the enhancing effect of erythrocytes (Fig. 2). However, overall activation fell short of that achieved by stimulating untreated platelets plus erythrocytes. These results pertain to aspirin as a therapeutic modality. If platelets receive sufficient stimulation to overcome ASA inhibition, erythrocytes still augment activation. Erythrocyte promotion of platelet reactivity may in part explain the more modest therapeutic effects of aspirin than expected on the basis of experiments with platelets alone.

Platelet–erythrocyte interactions in the presence of protease inhibitors. Since ASA-treatment and/or ADP removal did not abolish erythrocyte enhancement of platelet activation (Fig. 5), we extended and combined these experiments with protease inhibition. Association of protease activity with platelet activation is well documented (13–15). Proteolytic activity played a significant role during platelet–erythrocyte interactions with collagen (Table II). Protease inhibition reduced 5-HT release in platelet–erythrocyte suspensions, but not in collagen-stimulated platelets alone.

Table IV. Effects of Erythrocytes on Eicosanoid Profiles of Releasates from [^{3}H]Arachidonic Acid–labeled Platelets Activated in the Generating System, in the Presence of Aspirin

	12-HETE		AA	
Stimulus*	Platelets alone	Platelets + erythrocytes	Platelets alone	Platelets + erythrocytes
	cpm			
Collagen				
Mean	624	1,581‡	331	1,799§
SEM	193	468	68	369
Thrombin				
Mean	432	711‡	205	2,013§
SEM	74	91	51	424
Ionophore				
Mean	4,744	3,812	1,669	7,587‡
SEM	1,543	1,022	740	3,428

Agonists were added to [^{3}H]arachidonate-labeled platelets (2×10^8/ml) or to platelet–erythrocyte mixtures (40% hematocrit) in the presence of ASA (1 mM) in the generating system. Eicosanoids were isolated, separated, and quantified by thin-layer chromatography and scanning. Values are expressed as total counts per thin-layer chromatographic peak; n = 6–10.
* Collagen (16 μg/ml); thrombin (1 U/ml); ionophore (2 μM).
‡ $P < 0.025$; § $P < 0.005$ using paired t test (platelets vs. platelets plus erythrocytes).

Participation of protein kinase C in erythrocyte enhancement of platelet responsiveness was evaluated in preliminary experiments (data not shown), using staurosporine (1 μM) (16). After stimulation with each agonist, release of 5-HT from platelets alone was strongly inhibited, but in each instance erythrocytes enhanced platelet activation. Erythrocyte amplification also occurred when platelets were simultaneously treated with staurosporine, ASA, and enzymatic removal of ADP.

Thus, systematic treatment of platelet-erythrocyte mixtures with inhibitors of platelet activation, alone or in combination, was insufficient to abolish erythrocyte enhancement effects. We conclude that erythrocyte enhancement may involve not only known, but also as yet unidentified pathways of platelet activation.

Effect of erythrocytes on platelet arachidonate and EPA release. Erythrocytes strongly influenced release and metabolism of platelet arachidonate and EPA with agonist and substrate specificity. They induced a selective enhancement of platelet phospholipase activity and/or an overall increase in platelet activation, since, with collagen and thrombin, they increased release of both arachidonate and EPA.

In contrast, with ionophore erythrocytes did not enhance arachidonate or EPA release. Arachidonate or EPA may already have been fully mobilized, since ionophore induced three- to fivefold greater fatty acid release than collagen or thrombin in platelets alone (Tables III and V). However, platelet incorporation of arachidonate and EPA were similar, as was release induced by collagen and thrombin. In contrast, ionophore-induced release was 1.6-fold less for EPA than arachidonate (Tables III and V). This suggested that the releasable pool of EPA was not "metabolically exhausted." Alternatively,

Table V. Release of Radioactivity from [^{3}H]Eicosapentaenoic Acid-labeled Platelets or Platelet-Erythrocyte Suspensions Stimulated with Collagen, Thrombin, or Ionophore

Stimulus*	No aspirin		Aspirin	
	Platelets alone	Platelets + erythrocytes	Platelets alone	Platelets + erythrocytes
	dpm			
Collagen	10,798±1,534	20,093±1,556‡	7,585±1,079	10,628±1,095
Thrombin	10,467±321	13,824±1,069	6,000±142	9,531±961‡
Ionophore	29,440±3,229	26,638±1,703	18,621±2,132	18,398±1,568

Agonists were added to [^{3}H]arachidonate-labeled platelets (2×10^8/ml) or to platelet-erythrocyte mixtures (40% hematocrit) in the generating system. Values are mean±SEM of disintegrations per minute in 50 μl of releasate from activated cells. n = 4–6. * Collagen (16 μg/ml); thrombin (1 U/ml); ionophore (2 μM). ‡ $P < 0.005$ using paired t test (platelets vs. platelets plus erythrocytes).

ionophore could have acted on erythrocytes (17, 18), as well as platelets, thereby initiating eicosanoid metabolism in this cell-cell interaction via different mechanisms than collagen or thrombin.

As with 5-HT release, cyclooxygenase activity was not essential for erythrocyte promotion of arachidonate release. Furthermore, ADP removal did not impede erythrocyte enhancement of arachidonate release or subsequent metabolism (data not shown). Thus, two important pathways of platelet responsiveness are not critical components of the platelet-erythrocyte interactions reported here.

Eicosanoid metabolism of platelets alone. Stimulated platelets alone converted > 80% of released arachidonate or EPA to metabolites (Fig. 6, Table VI). TXB_2 and 12-HHTrE were the major arachidonate products with collagen and thrombin. Unexpectedly, ionophore activated lipoxygenase to a much greater extent than cyclooxygenase (Fig. 6). Two explanations seem plausible: (*a*) since much more substrate was released by ionophore, cyclooxygenase activity may have self-inactivated before 1 min (19, 20), permitting a greater degree of lipoxygenation; or (*b*) released arachidonate could have originated from different pools with different accessibilities to their metabolizing enzymes (21).

Released EPA was preferentially metabolized to 12-HEPE with each agonist, especially ionophore (Table VI). Increased conversion of EPA to 12-HEPE as compared with arachidonate to 12-HETE was reported (22, 23), and may be related to lesser affinity of cyclooxygenase for EPA than for arachidonate (22). Furthermore, lipoxygenase has a greater capacity for conversion of EPA than for arachidonate (23).

Stimulus-specificity of platelet eicosanoid profiles as regulated by erythrocytes. Our data demonstrate for the first time that intact erythrocytes regulate qualitative and quantitative production of eicosanoids by stimulated platelets in an agonist-specific manner. Since collagen is the first agonist with which platelets come into contact during hemostasis, the dramatic promotion of TXA_2 formation and arachidonate release and accumulation by erythrocytes at 1 min (Fig. 6) is important, because of their significant roles in platelet activation and recruitment (24). These early erythrocyte amplification events

Table VI. Effects of Erythrocytes on Eicosanoid Profiles of Releasates from [^{3}H]Eicosapentaenoic Acid-labeled Platelets Activated in the Generating System

Stimulus*	TXB_3		12-HHTE		12-HEPE		EPA	
	Platelets alone	Platelets + erythrocytes	Platelets alone	Platelets + erythrocytes	Platelets alone	Platelets + erythrocytes	Platelets alone	Platelets + erythrocytes
				cpm				
Collagen								
Mean	922	2,126‡	1,205	2,563‡	2,187	2,029	555	3,769‡
SEM	128	427	188	427	492	484	155	852
Thrombin								
Mean	829	962	963	1,145	1,811	1,538	776	3,576‡
SEM	73	112	90	178	158	307	239	514
Ionophore								
Mean	2,650	2,133	2,689	2,741	6,231	2,518‡	1,479	4,336‡
SEM	179	164	40	246	395	562	333	822

Agonists were added to [^{3}H]eicosapentaenoate-labeled platelets (2×10^8/ml) or to platelet-erythrocyte mixtures (40% hematocrit) in the generating system. Eicosanoids were isolated, separated, and quantified by thin-layer chromatography and scanning. Values are expressed as total counts per thin-layer chromatographic peak; n = 4–6. * Collagen (16 μg/ml); thrombin (1 U/ml); ionophore (2 μM). ‡ $P < 0.05$ using paired t test (platelets vs. platelets plus erythrocytes).

Table VII. Effects of Erythrocytes on Eicosanoid Profiles of Releasates from [^{3}H]Eicosapentaenoic Acid–labeled Platelets Activated in the Generating System, in the Presence of Aspirin

Stimulus*	12-HEPE		EPA	
	Platelets alone	Platelets + erythrocytes	Platelets alone	Platelets + erythrocytes
	cpm			
Collagen				
Mean	2,103	1,499	651	3,144‡
SEM	310	326	150	1,128
Thrombin				
Mean	1,421	1,092	514	2,158§
SEM	182	78	52	307
Ionophore				
Mean	10,321	3,591§	2,177	4,564‡
SEM	881	804	497	693

Agonists were added to [^{3}H]eicosapentaenoate-labeled platelets (2×10^8/ml) or to platelet-erythrocyte mixtures (40% hematocrit) in the presence of ASA (1 mM) in the generating system. Eicosanoids were isolated, separated, and quantified by thin-layer chromatography and scanning. Values are expressed as total counts per thin-layer chromatographic peak; $n = 4$–6.

* Collagen (16 μg/ml); thrombin (1 U/ml); ionophore (2 μM).

‡ $P < 0.05$; § $P < 0.005$ using paired t test (platelets vs. platelets plus erythrocytes).

support the concept of direct participation of erythrocytes in hemostasis. They can be correlated with clinical observations that bleeding times are prolonged in anemia and corrected by normalization of the hematocrit (9), although rheologic effects may also play a role in in vivo situations.

Collagen-stimulated cell mixtures generated more than twice the TXA_2 and 12-HHTrE than did thrombin, despite similarly enhanced availability of free arachidonate. The high ratio between free arachidonate and TXB_2 induced by thrombin as compared to collagen demonstrates an erythrocyte-dependent, agonist-specific effect on platelet cyclooxygenase. A similar effect occurred with EPA metabolism.

After ASA treatment, erythrocytes resulted in greater 12-HETE production with collagen than thrombin, but arachidonate accumulation was similar, again emphasizing stimulus specificity of the erythrocyte effect (Fig. 6, Table IV).

Erythrocytes modulated platelet eicosanoid production in a unique manner with ionophore (Fig. 6). Releasates from ionophore-stimulated platelets alone contained far greater quantities of eicosanoids (more than threefold), than those from collagen- or thrombin-activated platelets. Nevertheless, no amplification of platelet eicosanoid formation by erythrocytes occurred, despite availability of free arachidonate. In fact, erythrocytes markedly reduced 12-HETE formation with ionophore (Fig. 6). Since free arachidonate was available, erythrocytes apparently inhibited lipoxygenation. Unmetabolized arachidonate was increased by erythrocytes with each agonist—the "hallmark effect" of our experiments. When product formation was examined as percent of total substrate released, a relative inhibition of lipoxygenation by erythrocytes was discernible with each agonist. This could have contributed to increased accumulation of unmetabolized arachidonate, which would then be available for platelet recruitment and transcellular metabolism (5).

Effects of erythrocytes on platelet EPA metabolism. Modulation of eicosanoid formation from EPA by erythrocytes reflected substrate differences from arachidonate. Notably, with or without ASA, erythrocytes induced a decrease in 12-HEPE formation with all agonists. 12-HEPE has been proposed as an anti-inflammatory and anti-thrombotic fish oil metabolite (25, 26). Reduction of 12-HEPE might counteract some of the beneficial effects of EPA. On the other hand the increase in TXB_3 with collagen and thrombin may be therapeutically beneficial (27–29).

The pronounced enhancement of free EPA accumulation (Tables VI, VII) is of functional importance since EPA, in contrast to arachidonate is not proaggregatory (29, 30). Free EPA competes with arachidonate and leads to production of eicosanoids with different biological properties. Erythrocyte-induced enhancement of free EPA accumulation will therefore contribute to the action of EPA as an anti-thrombotic and anti-inflammatory agent.

Activation and recruitment as separate expressions of platelet reactivity. Although activation and recruitment often occur in parallel, there was a wide divergence between these two parameters in at least three experimental settings. (*a*) Upon ASA treatment and ionophore stimulation, activation far exceeded recruitment (Figs. 2, 4). Although arachidonate in the releasate should, theoretically, have contributed to recruitment, it did not do so in a major way. This was demonstrated in a comparison of assay systems which were either ASA treated, or ASA free (data not shown). We conclude from the results in Fig. 4 that, in the case of ionophore, arachidonate transfer was not sufficient to stimulate the platelets in the assay system. We speculate that thrombin- and collagen-activated releasates contain a component necessary to induce recruitment, which is not present in ionophore-activated releasates (Fig. 4). Alternatively, ionophore stimulation may directly or indirectly yield an inhibitory component. (*b*) When ADP was enzymatically removed, activation by collagen and thrombin was minimally affected, but recruitment was greatly reduced (Table I). (*c*) With collagen, erythrocyte enhancement was dose-dependent. At 4 μg/ml enhancement of recruitment was proportionately greater than at 16 μg/ml (Figs. 3 and 4), but the reverse was true for activation (data not shown).

Clinical implications of these data relate to interpretations of measurements of platelet activation in body fluids (eg metabolites of eicosanoids such as TXA_2). Elevated levels have been correlated with clinical thrombotic events. If activation occurred with minimal recruitment, these correlations may require re-evaluation.

In conclusion, we developed a two-stage in vitro system to study initial events in human platelet activation and recruitment independently. These two phenomena do not always occur in parallel. Cell-free releasates from platelets in the presence of erythrocytes had greater recruitment capacity than those from stimulated platelets alone. Intact erythrocytes in the presence of activated platelets: first, enhanced platelet activation, even in the setting of aspirin treatment, ADP removal and/or protease inhibition; secondly, increased platelet arachidonate or eicosapentaenoate mobilization after collagen or thrombin stimulation, with or without aspirin or ADP re-

moval; thirdly, modulated cyclooxygenase and lipoxygenase metabolite formation in a stimulus-specific manner; and fourthly, increased accumulation of free arachidonate or eicosapentaenoate in the cellular environment. These studies provide a metabolic basis for the recognized participation of erythrocytes in hemostasis and thrombosis, and emphasize the importance of considering these processes as multicellular events.

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