Intravascular Filarial Parasites Inhibit Platelet Aggregation

Role of Parasite-derived Prostanoids

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Abstract

The nematode parasites that cause human lymphatic filariasis survive for long periods in their vascular habitats despite continual exposure to host cells. Platelets do not adhere to blood-borne microfilariae, and thrombo-occlusive phenomena are not observed in patients with circulating microfilariae. We studied the ability of microfilariae to inhibit human platelet aggregation in vitro. *Brugia malayi* microfilariae incubated with human platelets caused dose-dependent inhibition of agonist-induced platelet aggregation, thromboxane generation, and serotonin release. As few as one microfilaria per 10^4 platelets completely inhibited aggregation of platelets induced by thrombin, collagen, arachidonic acid, or ionophore A23187. Microfilariae also inhibited aggregation of platelets in platelet-rich plasma stimulated by ADP, compound U46619, or platelet-activating factor. The inhibition required intimate proximity but not direct contact between parasites and platelets, and was mediated by parasite-derived soluble factors of low (<1,000 M_r) molecular weight that were labile in aqueous media and caused an elevation of platelet cAMP. Prior treatment of microfilariae with pharmacologic inhibitors of cyclooxygenase decreased both parasite release of prostacyclin and PGE_2 and microfilarial inhibition of platelet aggregation. These results indicate that microfilariae inhibit platelet aggregation, via mechanisms that may include the elaboration of anti-aggregatory eicosanoids. (*J. Clin. Invest.* 1992. 89:1113–1120.) Key words: filariasis • nematodes • helminths • prostacyclin • prostaglandins

Introduction

The remarkable ability of filarial parasites to persist intravascularly in the presence of host blood cells has puzzled investigators of parasitic disease (1, 2). In lymphatic filariasis, adult worms inhabit lymphatic vessels, and release offspring microfilariae, which enter the bloodstream, where they may survive for several months or more. The lymphatic pathology in this disease is largely attributable to the adult worms. Although a brisk immune response to microfilariae occurs in the syndrome of tropical pulmonary eosinophilia, in most patients the presence of microfilariae in the blood is not associated with specific disease manifestations; in fact, most microfilaremic patients are asymptomatic (3). Although microfilariae may repeatedly encounter host platelets and the vascular wall, platelets do not adhere to microfilariae. Furthermore, microfilariae are not usually irreversibly trapped within small blood vessels, despite their relatively large size (~ 220 μm long by ~ 10 μm diameter). Indeed, microvascular occlusion and thromboembolic phenomena characteristically do not occur in infected microfilaremic persons, even those with high-grade microfilaremias in excess of 10,000 microfilariae per milliliter of blood. The interaction between microfilariae and platelets is also of considerable importance to the parasite, because unrestricted circulation of microfilariae is critical for the transmission of filarial infections by biting mosquito vectors. The capacity of live microfilariae to affect platelet aggregation and other normal platelet responses has not been previously studied in vitro. We have previously demonstrated that microfilariae of *Brugia malayi* use arachidonic acid to generate prostacyclin and PGE_2, which are released into the medium surrounding the parasites (4–6). These findings suggested that microfilariae might modulate platelet responses via the release of these anti-aggregatory prostanoids. We therefore examined the ability of microfilariae to inhibit platelet aggregation and assessed the potential role of parasite-derived prostanoids in this process.

Methods

Reagents. Potato apyrase (grade VIII), bovine thrombin, ADP, platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, PAF),[1] adenosine deaminase (type V), 3-isobutyl-1-methylxanthine, N^G-methyl-L-arginine, $N\omega$-nitro-L-arginine, indomethacin, and arachidonic acid (Sigma Chemical Co., St. Louis, MO), [5,8,11,14]-eicosatetraynoic acid (ETYA) (Biomol Research Laboratories, Inc.; Plymouth Meeting, PA), calcium ionophore A23187 (Calbiochem-Behring Corp., La Jolla, CA), collagen (Hormone-Chemie, Munich, Germany), and the PGH_2 analogue U46619 (Upjohn Co., Kalamazoo, MI) were purchased from the sources indicated. Iloprost, a stable prostacyclin analogue, was a gift from Dr. E. Schillinger (Schering AG, Berlin, Germany). Compound BW755c (3-amino-1-(3-trifluoromethylphenyl)-2-pyrazoline) was a gift from Dr. S. Moncada (Wellcome Research Laboratories, Beckenham, Kent, UK).

Isolation of microfilariae. Jirds infected intraperitoneally with *B. malayi* were provided by the Filariasis Repository of the University of Georgia, Athens. Microfilariae were obtained from ketamine-anesthetized jirds by saline peritoneal lavage, and separated from jird peritoneal cells by passage over a Sephadex G-25 column as described (6).

Preparation of platelet suspensions. Blood from healthy donors who had not taken any medications for at least 2 wk was drawn by veni-

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1. *Abbreviations used in this paper:* ETYA, 5,8,11,14-eicosatetraynoic acid; PAF, platelet-activating factor; PRP, platelet-rich plasma.

puncture into 0.1 vol of 3.8% sodium citrate pH 6.5. After anticoagulated whole blood was centrifuged (200 *g* for 15 min), platelet-rich plasma was collected. For some experiments, citrated platelet-rich plasma was used directly. To prepare washed platelets, platelet-rich plasma was spun at 1,000 *g* for 10 min. The platelet pellet was resuspended and washed twice with HBSS containing 1 mM EGTA and 1 U/ml apyrase, and finally suspended in incubation buffer which was a modified Hepes-Tyrode's solution containing 123 mM NaCl, 8.3 mM sodium bicarbonate, 2.8 mM KCl, 0.8 mM KH_2PO_4, 5.6 mM dextrose, 1 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 10 mM Hepes, pH 7.4, and enumerated using a Coulter counter (model ZF; Coulter Electronics, Inc., Hialeah, FL). For experiments in which platelets were pretreated to inhibit platelet prostanoid formation, platelet-rich plasma was incubated with 1 mM aspirin at room temperature for 30 min before the wash steps. The effectiveness of aspirin treatment of platelets was verified by HPLC and RIA, which indicated > 97% reduction in platelet thromboxane generation compared to untreated platelets. For some experiments assessing platelet-derived prostanoids, washed platelets were prelabeled with [^{14}C]arachidonic acid (20 μM, 55 mCi/mmol; Amersham Chemical Corp., Arlington Heights, IL) in 1 ml of Hepes-Tyrode's buffer at 37°C for 60 min, then washed three times with buffer before incubations.

Coincubation of microfilariae and platelets. Washed platelets (2.5×10^8) and varying numbers of microfilariae (0–2.5×10^5) were coincubated in 1 ml of the Hepes-Tyrode's solution in polypropylene microfuge tubes (Treff AG, Degersheim, SW). To bring platelets and microfilariae into direct contact, platelets and microfilariae were first pelleted by centrifugation at 735 *g* for 10 min in an Eppendorf microfuge (Brinkman Instruments, Inc., Westbury, NY). Less than 1% of the microfilariae and < 5% of the platelets remained suspended in the medium after this centrifugation. After incubation for 60 min in a 37°C water bath, the entire pellet containing platelets and microfilariae was gently resuspended. The microfilariae were then sedimented by centrifugation at 80 *g* for 3 min, which allowed > 98% of the platelets to remain in suspension. For platelet aggregation assays, 0.4-ml aliquots of the platelet suspension (containing 1×10^8 platelets and < 200 microfilariae) were sampled. Platelets incubated with heat-killed microfilariae (2.5×10^5 microfilariae boiled for 5 min, then washed twice with incubation buffer) or no microfilariae, and taken through identical centrifugation steps before and after the incubation period, served as controls. For some samples, platelets and microfilariae were not pelleted at the beginning of the incubation but allowed to remain in suspension, followed by selective sedimentation of microfilariae at the end of the incubation period. For incubations of microfilariae in platelet rich plasma, 2.5×10^5 microfilariae were added to 1 ml of citrated platelet-rich plasma containing ~ 2.5–4.0 $\times 10^8$ platelets/ml. The microfilariae and platelets were then pelleted and incubated, with subsequent separation of platelets and parasites, as above.

Platelet aggregation. Platelet aggregation after the addition of 0.1 U/ml of thrombin was measured by standard turbidimetric techniques (7), in which 0.4-ml aliquots of platelets were stirred at 1,000 rpm at 37°C in a dual-channel aggregometer (Sienco, Morrison, CO). Other agonists used to aggregate washed platelets included 2 μg/ml collagen, 1 μM arachidonic acid, and 1 μM calcium ionophore A23187. For platelet-rich plasma, threshold doses of ADP (2.5 μM), U46619 (0.5 μM), and PAF (0.25 μM), each resulting in a primary aggregation response, were used as agonists (7). Percent aggregation was calculated as the percentage of full scale light transmission achieved 4 min after addition of agonist.

Platelet thromboxane generation. To assess thromboxane release following the addition of thrombin as an agonist, platelet thromboxane A_2 generation was terminated by the addition of ice-cold indomethacin (10 μM) directly into the aggregation cuvette. The quenched sample was kept on ice, and the media removed after centrifugation at 14,000 *g* for 2 min. Concentrations of thromboxane B_2, the stable hydrolysis product of thromboxane A_2, were determined by using a RIA kit (Advanced Magnetics, Inc., Cambridge, MA).

Platelet [^{14}C]serotonin loading and release. Platelets were loaded with radiolabeled serotonin by the addition of 2 μCi of 5-[2-^{14}C]hydroxytryptamine binoxalate (50 mCi/mmol; New England Nuclear, Boston, MA) to 20 ml of platelet-rich plasma and incubation at 37°C for 30 min before the wash steps. The final radioactivity was ~ 20,000 cpm/ml of washed platelets. After incubations with or without microfilariae, [^{14}C]serotonin-loaded platelets were stirred in the aggregometer in the presence of thrombin as above. Platelet secretory granule release was terminated after 4 min by the addition of an equal volume of ice-cold 4% paraformaldehyde and 10 mM EDTA. After centrifugation at 14,000 *g* for 2 min, ^{14}C-radioactivity was measured in the supernatant by liquid scintillation counting. These measurements represent a minimum estimate of secretion due to potential serotonin reuptake.

Effect of coincubation medium alone on platelet aggregation. Media (1 ml) was removed after a 60-min incubation of microfilariae (2.5×10^5) alone, or microfilariae and platelets (2.5×10^8), and kept at 37°C for varying time periods. At different time points ranging from 30 s (the shortest feasible time) to 30 min after removal, 0.4-ml aliquots of the standing media were then added directly to pellets of newly washed platelets (1×10^8). Aggregation of these platelets suspended in incubation media was then measured as described above, and compared to that of control platelets incubated in fresh Hepes-Tyrode's solution.

Incubation of microfilariae and platelets in separate compartments. A two-compartment co-culture system (Transwell; Costar Corp., Cambridge, MA) was used for experiments requiring the physical separation of microfilariae and platelets during incubation. In this apparatus, 6.5-mm Transwell inserts with 0.4-μm-pore size polycarbonate membrane bottoms fit inside culture wells of a 24-well polystyrene cluster plate, with a distance between the bottom of the porous membrane and the bottom of the culture well of 1 mm. Suspensions of parasites containing 1.5×10^5 microfilariae in 0.1 ml Hepes-Tyrode's solution were placed in each Transwell insert, and washed platelet suspensions containing 1.5×10^8 platelets in 0.6 ml buffer were added to the outer culture well. Incubations were carried out at 37°C for 60 min with gentle shaking (10 rpm) of the cluster plate. After incubation, 0.4-ml aliquots of the platelet suspensions in the outer wells were removed for aggregation studies. For some samples, adenosine deaminase (2 U/ml) or 3-isobutyl-1-methylxanthine (2 μM) were added to the outer well containing platelets (8). For some experiments, the 0.4-μm-pore size polycarbonate membranes were excised and replaced by porous cellulose membranes of exclusion sizes M_r 30,000, 12,000–14,000, and 1,000 (Spectra/por; Spectrum Medical Industries, Inc., Los Angeles, CA). Each porous cellulose membrane was first equilibrated in Hepes-Tyrode's incubation buffer, cut to size, and held in place on the Transwell insert with a 9-mm i.d., tight-fitting polypropylene ring fashioned from the top of a microfuge tube.

Measurement of platelet cyclic nucleotides. The Transwell apparatus was similarly employed in experiments to assess platelet cAMP and cGMP responses, except that numbers of microfilariae were varied, and 3-isobutyl-1-methylxanthine (2 μM) was added as a phosphodiesterase inhibitor. Resting platelet cAMP and cGMP levels were measured after 60-min incubations of washed platelets (1×10^8) with microfilariae (0–1.5×10^5). For some samples, 5 nM iloprost or 0.4% (vol/vol) nitric oxide were added in lieu of microfilariae. Nitric oxide was prepared just before use by mixing equal volumes of 300 mM sodium nitrite and 50 mM sodium acetate, pH 3.5 in deoxygenated water (9). Ice-cold TCA (final concentration 10%) was added to 0.1-ml aliquots of platelets immediately after incubations. The soluble extract was removed after centrifugation at 6,000 *g* for 3 min at 4°C, then washed five times with water-saturated diethyl ether to remove TCA (10). Concentrations of platelet cAMP and cGMP were determined using RIA kits (Advanced Magnetics, Inc., Cambridge, MA) with an acetylation modification (10).

Treatment of microfilariae with pharmacologic inhibitors of arachidonic acid metabolism. To assess the role of microfilarial prostanoid formation in parasite–platelet interactions, equal numbers of microfilariae were first incubated with or without inhibitors of prostanoid

biosynthesis in 1 ml of Hepes-Tyrode's solution at 37°C for 60 min, then washed three times with buffer. These agents included aspirin (100 μM), indomethacin (100 μM), ETYA (20 μM), and BW755c (100 μM) (6). Microfilarial viability, assessed by motility under light microscopy, was ≥ 97% after treatment with these agents. Platelet prostanoid biosynthesis was blocked by prior treatment with aspirin as described above. Platelets (1×10^9) were coincubated for 60 min with treated or control untreated microfilariae (1×10^6) in microfuge tubes as described above, and aliquots of platelets were then removed for aggregation studies. The effectiveness of pharmacologic inhibition was determined in separate experiments in which microfilariae were pretreated as above for 60 min, then incubated with [^{14}C]arachidonic acid (50 μM) at 37°C for 60 min. Lipids in incubation media were extracted and resolved by TLC as described (5, 6), and radioactive peaks were detected by a TLC radiation scanner (model RS; Radiomatic Instruments, Tampa, FL). Migrations of microfilarial ^{14}C-labeled prostanoids were compared with cochromatographed 6-keto-$PGF_{1\alpha}$ and PGE_2 (5), and peak areas were calculated using the manufacturer's software program.

Results

Microfilarial inhibition of platelet aggregation. To assess the effect of microfilariae on platelet aggregability, parasites were incubated with isolated human platelets. In this initial series of experiments, microfilariae were sedimented with the platelets to induce maximal cell contact. A 60-min incubation of normal human platelets in contact with increasing numbers of microfilariae resulted in dose-dependent inhibition of platelet aggregation stimulated by thrombin, a standard agonist for in vitro platelet aggregation (Figs. 1 and 3 *A*). Tracings in Fig. 1 show that exposure of platelets to parasites at a ratio of 1 microfilariae per 10^4 platelets resulted in complete inhibition of thrombin-induced aggregation, whereas microfilariae at a greater ratio of 1 microfilaria per 10^3 platelets blocked agonist-induced platelet shape change (i.e., no initial decrease in amplitude of the baseline) as well as aggregation. The aggregation of washed platelets to other standard agonists was also affected: after 60 min incubation with microfilariae (microfilariae/platelet ratio 1:10^3), platelet aggregation to stimulatory doses of collagen (2 μg/ml), arachidonic acid (1 μM), and ionophore A23187 (1 μM) was completely inhibited (not shown). Similar results were obtained using platelets treated with aspirin (not shown). Platelets were not adherent to the motile living worms when viewed under light microscopy. Platelet aggregation after incubation with heat-killed microfilariae or buffer alone was normal (Fig. 1).

To examine the effects of microfilariae on platelets in intact plasma, parasites were sedimented in platelet-rich plasma (2.5×10^5 microfilariae per ml) as above. Because thrombin is not a reliable agonist for platelets in plasma due to plasma thrombin inhibitors (7) and in vitro clot formation, several other aggregating agents were used which cause aggregation of citrated platelet rich plasma: ADP, the PGH_2 analogue U46619, and PAF. For each of these agonists, a threshold dose which resulted in primary reversible aggregation of control PRP was used (Fig. 2, *left*). The addition of microfilariae resulted in suppression of primary aggregation induced by ADP, U46619, and PAF (Fig. 2, *right*). Plasma enzymes that break down ADP reverse, but do not block, primary aggregation (7), and the addition of 2 U/ml apyrase, an exogenous ADPase, did not block ADP-induced primary aggregation of PRP (not shown).

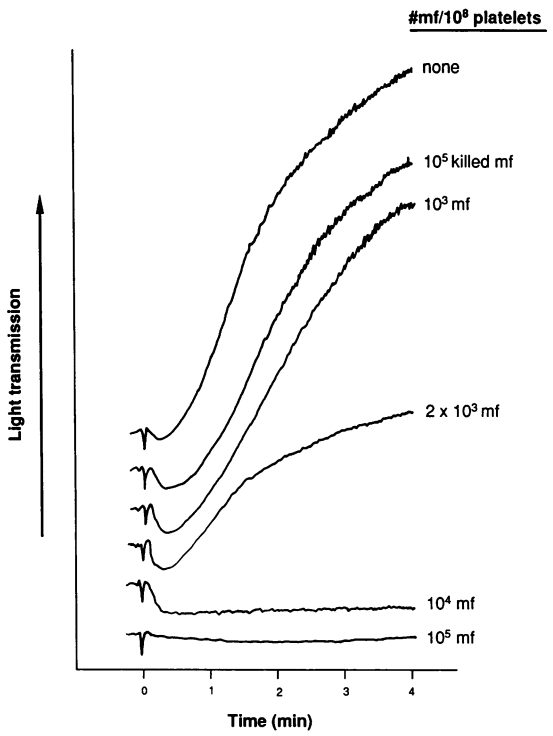

Figure 1. Inhibition of aggregation of isolated human platelets by *B. malayi* microfilariae. Platelet aggregation was measured turbidimetrically after the addition of thrombin 0.1 U/ml at time 0 min to washed human platelets (2.5×10^8) that had been recovered after 60 min incubation in contact with varying numbers ($0–2.5 \times 10^5$) of microfilariae (*mf*). Representative platelet aggregation tracings after incubations with different numbers of microfilariae are shown superimposed. Thrombin-induced platelet aggregation was completely suppressed with 10^4 mf/10^8 platelets and was partially inhibited by 2×10^3 mf/10^8 platelets, whereas 10^5 heat-killed mf/10^8 platelets did not affect platelet aggregation. At a greater ratio of 10^5 mf/10^8 platelets, thrombin-induced platelet shape change as well as aggregation were inhibited. Similar results were obtained using collagen, arachidonic acid, or calcium ionophore A23187 (not shown).

To further assess the microfilarial inhibition of platelet activation, agonist-induced platelet thromboxane A_2 generation and serotonin release were measured. As shown in Figs. 3 *B* and *C*, increasing numbers of microfilariae caused progressive inhibition of platelet thromboxane generation and serotonin release, in concert with inhibition of aggregation.

Mechanisms of parasite inhibition of platelet aggregation. Platelet aggregability was unaffected when washed platelets and microfilariae were allowed to remain free in suspension, without close contact achieved by pelleting both platelets and microfilariae at the start of the incubation (percent aggregation and maximal slope of aggregation not different from control platelets, data not shown). To determine if direct physical contact was required for parasite inhibition of platelet aggregation, we used a dual-chamber culture apparatus in which microfilariae and washed platelets (microfilariae/platelet ratio 1:10^3) were incubated in separate compartments ~ 1 mm apart with a 0.4-μm-pore membrane between the two chambers. As shown in Fig. 4, platelets incubated for 60 min in such proximity to microfilariae failed to aggregate in response to thrombin, indicating the presence of soluble mediators passing from the parasites through the permeable membrane to the platelets.

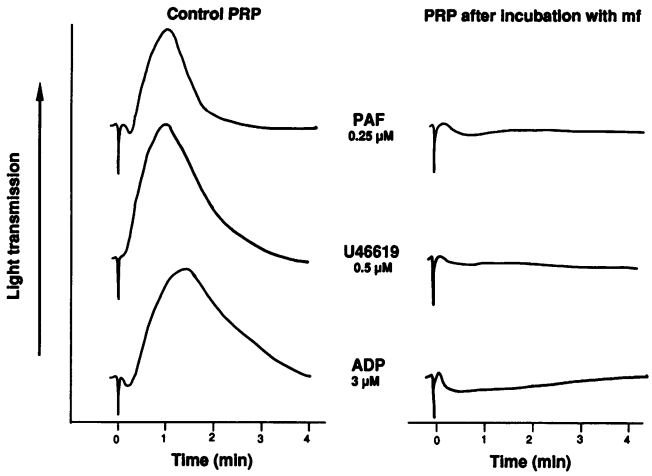

Figure 2. Microfilarial inhibition of platelet aggregation in platelet rich plasma. Parasites were incubated (2.5×10^5 microfilariae per ml) in platelet-rich plasma as in Fig. 1. The threshold dose of ADP, compound U46619, and PAF, resulting in primary reversible aggregation of control platelet-rich plasma (*PRP*), was determined (*left*). Identical doses of agonists added to platelet rich plasma recovered after incubation with microfilariae (*mf*) did not result in primary aggregation (*right*). Representative tracings from three separate experiments are shown.

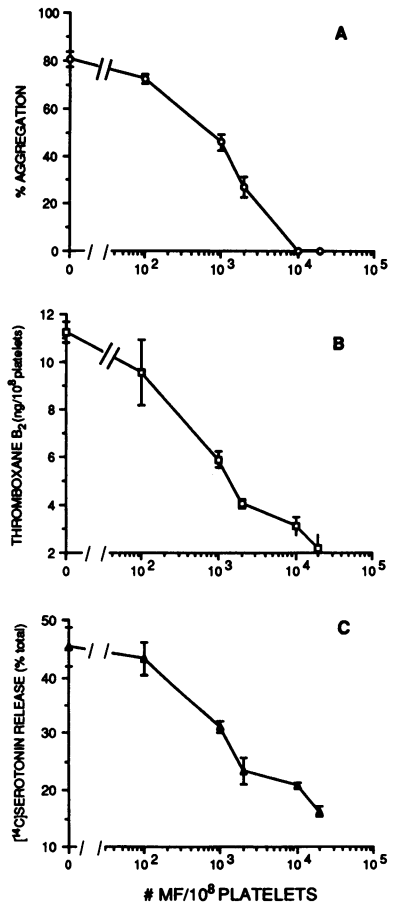

Figure 3. Microfilarial dose dependent inhibition of platelet responses after 60 min incubation with microfilariae. The percent aggregation of washed platelets after incubations with increasing numbers of microfilariae (*A*) was calculated from the maximal extent of aggregation achieved in the 4-min period after thrombin addition. Platelet thromboxane A_2 generation (*B*) was measured as immunoreactive thromboxane B_2 in samples quenched 4 min after thrombin stimulation by addition of indomethacin and centrifugation. Using [^{14}C]serotonin-loaded platelets in similar experiments, platelet serotonin release (*C*) was measured via liquid scintillation counting in supernatants 4 min after thrombin addition by terminating release with 4% paraformaldehyde and EDTA followed by centrifugation. Data represent the means±SEM of six experiments for *A* and three experiments each for *B* and *C*.

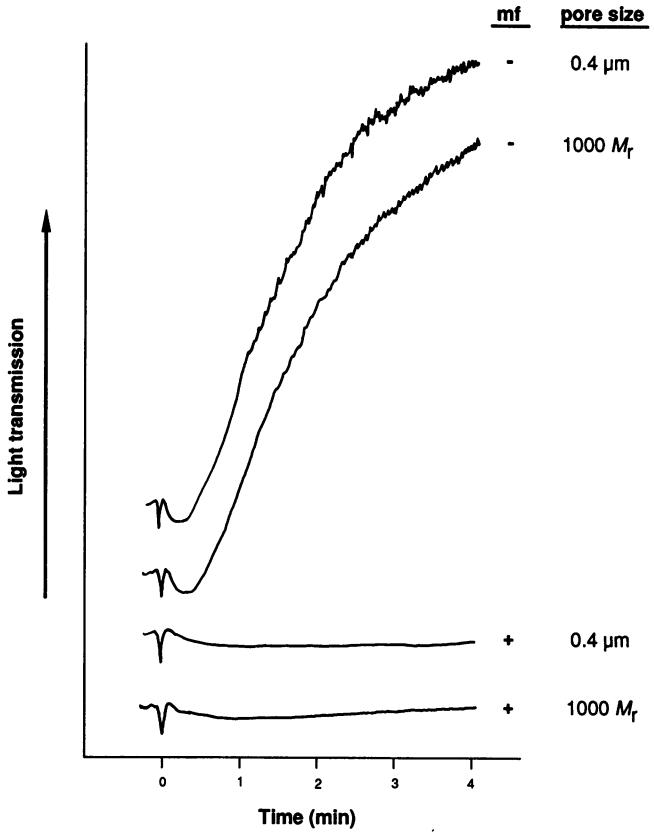

Figure 4. Inhibition of platelet aggregation by microfilariae separated from platelets by permeable membranes. Washed platelets were incubated with (+) or without (−) microfilariae (microfilariae:platelet ratio $1{:}10^3$) in a Transwell co-culture system at 37°C for 60 min using permeable membranes of the indicated pore size or exclusion size to separate the parasites and platelets. Tracings of platelet aggregation responses to 0.1 U/ml thrombin added at time 0 min are shown, and are representative of three experiments. Incubations of microfilariae and platelets using permeable membranes of exclusion sizes 25,000 M_r and 12,000–14,000 M_r resulted in completely inhibition of platelet aggregation (not shown) similar to that shown using permeable membranes of exclusion size 1,000 M_r.

Platelets incubated with heat-killed microfilariae (not shown) or no microfilariae exhibited normal aggregation (Fig. 4). Inhibition of platelet aggregation was time-dependent: after 15 min incubation with microfilariae in this system, thrombin-induced platelet aggregation was inhibited 50%; after 30 min, platelet aggregation was completely inhibited; after 60 min (Fig. 4), platelet shape change was abolished as well. Adenosine deaminase (2 U/ml) or 3-isobutyl-1-methylxanthine (2 μM), added to the incubation to test whether adenosine contributed to inhibition, had no effect on agonist-induced platelet responses after exposure to microfilariae (not shown).

To estimate the relative size of the soluble parasite-derived mediators involved in the observed inhibition of platelet aggregation, dialysis membranes with exclusion sizes of 30,000, 12,000–14,000, and 1,000 M_r were substituted for the 0.4-μm-pore size membrane separating the platelets from microfilariae. Inhibition of platelet aggregability in the presence of microfilariae was maintained across all of these permeable membranes with exclusion sizes including 1,000 M_r (Fig. 4).

To assess the stability and transferability of the soluble anti-aggregatory property derived from microfilariae, incubation medium alone, obtained after 60 min coincubation of parasites and platelets in microfuge tubes, was removed and added to newly isolated platelets. Fresh platelets suspended in incubation medium that was transferred almost immediately (30 s) after removal did not aggregate significantly in response to thrombin stimulation. However, most of this inhibitory effect diminished rapidly over time that the medium was allowed to stand before its addition to fresh platelets, and by 2 min after removal the medium had only a modest inhibitory effect on platelet aggregation (76% of control platelet aggregation) (Fig. 5). Approximately 10% of the inhibitory activity of the medium was not rapidly labile within 30 min (Fig. 5). Similar results were obtained using media from the incubation of microfilariae alone without platelets (not shown). These results indicate that the parasite-derived soluble inhibitory factors in large part were highly labile, with rapid loss of activity in aqueous media at 37°C.

To determine if microfilarial inhibition of platelet responses was associated with increases in platelet cyclic nucleotide levels, platelet-associated cAMP and cGMP were measured after exposure to soluble products of microfilariae. Incubations were carried out in the dual chamber co-culture system, which also prevented any potential contribution from microfilariae to measured cyclic nucleotide levels. As shown in Fig. 6 *A*, resting platelet cAMP levels increased progressively in a dose-dependent manner after incubation with increasing numbers of microfilariae. Platelets incubated with the stable prostacyclin analogue iloprost at a dose of 5 nM, a concentration which inhibited platelet shape change and aggregation (not shown), served as a positive control for elevated platelet cAMP levels (Fig. 6 *B*). Levels of platelet cGMP following exposure to microfilariae (up to 10^5 microfilariae/10^8 platelets) were not elevated compared to platelets incubated without parasites (0.8 pmol/10^9 platelets). Platelets exposed to 0.4% nitric oxide, which blocked platelet shape change and aggregation (not shown), developed 10-fold elevated cGMP levels (8.4 pmol/10^9 platelets). Pretreatment of microfilariae with N^G-methyl-L-arginine (30–300 μM) or $N\omega$-nitro-L-arginine (30 μM), which are competitive inhibitors of nitric oxide formation from L-arginine (11, 12), did not affect parasite inhibition of platelet aggregation (not shown).

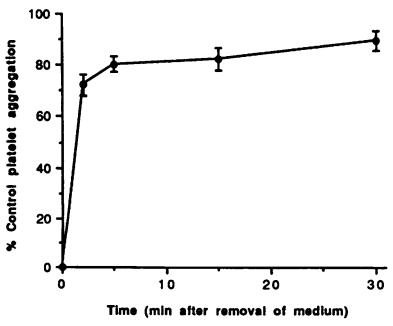

Figure 5. Lability of parasite-derived inhibitory factors in aqueous media assessed by effect of incubation media alone on platelet aggregation. After microfilariae (2.5×10^5) and platelets (2.5×10^8) were coincubated for 60 min, media was removed and held at 37°C for the indicated time periods, then added to fresh platelets. Agonist-induced platelet aggregation is expressed as percent (means±SE of duplicates) of maximal aggregation of control platelets incubated in buffer, and is plotted against the time period the medium was allowed to stand after removal.

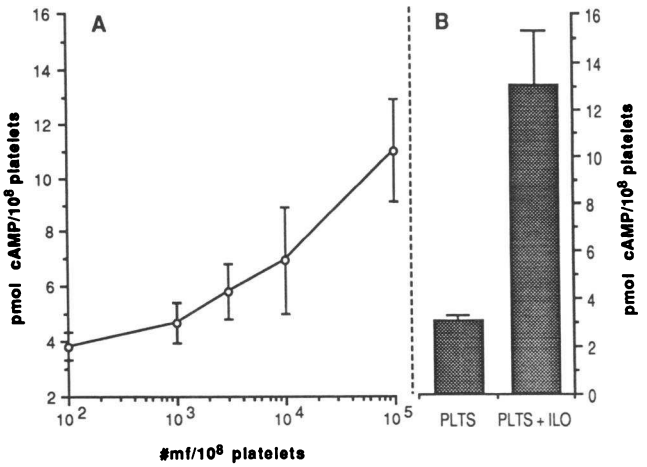

Figure 6. Microfilarial dose dependent elevation of platelet cAMP. Platelets (1.5×10^8) were incubated with microfilariae (0–1.5×10^5) for 60 min at 37°C in a Transwell co-culture system in the presence of 3-isobutyl-1-methylxanthine (2 μM) to block phosphodiesterase activity. *A* shows resting platelet cAMP levels after incubation with increasing numbers of microfilariae. *B* shows cAMP levels of control platelets incubated in buffer alone (*PLTS*) and platelets incubated with the stable prostacyclin analogue iloprost (5 nM) (*PLTS + ILO*). Platelet cAMP concentrations were measured by RIA after extraction, and represent the means±SEM of four experiments.

Role of microfilarial prostanoid release in inhibition of platelet aggregation. To determine if microfilarial prostanoid release contributed to parasite inhibition of platelet aggregation, microfilariae were treated with inhibitors of enzymatic prostanoid formation before incubation with platelets. Indomethacin, ETYA, and compound BW755c have been previously demonstrated to block microfilarial prostanoid formation (6). Pretreatment of microfilariae with either aspirin or these agents blocked the capacity of the parasites to inhibit platelet aggregation (Fig. 7); i.e., platelet aggregation after incubation with treated microfilariae approached that of control platelets incubated without parasites. The effectiveness of treatment with these agents was determined by prelabeling similar numbers of microfilariae with [^{14}C]arachidonic acid (50 μM) and thin-layer chromatography analysis of released prostanoids from treated or untreated microfilariae. Microfilarial ^{14}C-labeled prostanoid formation was significantly reduced by all of these agents, ranging from 79% inhibition by aspirin to 87% inhibition by ETYA, in comparison to untreated microfilariae.

Discussion

This study was designed to examine the effect of intact living microfilariae on the function of human platelets, and the possible role of parasite-derived prostanoids in mediating this effect. Our results indicate that microfilariae of *B. malayi* inhibit the aggregation of human platelets in a potent manner which is dependent, in part, on the elaboration of anti-aggregatory eicosanoids by the parasites.

Microfilariae sedimented with platelets exerted a striking and potent effect on platelet aggregability. Under these experimental conditions, a single microfilaria per 10^4 platelets was sufficient to completely suppress thrombin-induced platelet

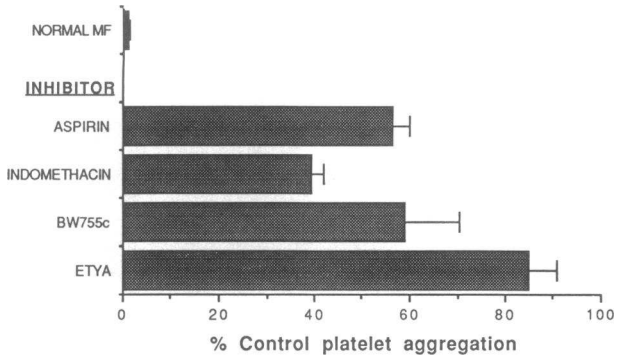

Figure 7. Effect of pretreatment of microfilariae with cyclooxygenase inhibitors on parasite inhibition of platelet aggregation. Equal numbers of microfilariae (1×10^6) were pretreated with aspirin (100 μM), indomethacin (100 μM), BW755c (100 μM), ETYA (20 μM), or buffer alone (*NORMAL MF*), washed, then incubated for 60 min with aspirin-treated platelets (1×10^9). Thrombin-induced aggregation is expressed as percent (means±SEM of three experiments) of maximal aggregation of control platelets incubated in buffer.

aggregation, while one microfilaria per 10^3 platelets suppressed platelet shape change as well. Conventional quantitative indicators of platelet activation, including platelet thromboxane generation and serotonin release, were likewise inhibited by microfilariae in a potent dose-dependent fashion. These parasite effects were not due to neutralization of the thrombin agonist itself (e.g., by secretion in vitro of a hirudin-like substance) since the aggregation of isolated platelets was similarly suppressed to diverse agonists including collagen, arachidonic acid, and calcium ionophore. Furthermore, these anti-platelet effects were also observed using platelet-rich plasma instead of washed platelets, wherein microfilariae suppressed aggregation induced by ADP, the PGH_2 analogue U46619, or platelet activating factor. Thus, although plasma might be expected to contain host factors which would counteract parasite metabolism, the suppressive effect of parasites was retained in this more physiological milieu. Because a wide variety of agonists (which act by different mechanisms) were used, the inhibitory effect of microfilariae appeared to be predominantly on intrinsic platelet responsiveness, rather than via agonist-specific effects.

Living microfilariae were required for the inhibition of platelet aggregation, which was unaffected following contact with heat-killed microfilariae. Although platelets in direct contact with microfilariae were inhibited, platelets $\sim$ 1 mm proximate to microfilariae but separated by a porous membrane were also inhibited. Thus, the capacity of microfilariae to inhibit platelet aggregation under these conditions could be accounted for by the metabolic release of soluble parasite-derived factors of low molecular weight ($\leq$ 1,000 M_r). Supernatant fluid (taken after 60 min incubation when inhibition of platelet aggregation and shape change were maximal) contained inhibitory activity if assayed immediately, but most activity was lost within minutes, indicating that the parasite-derived paracrine factors were in large part labile and did not accumulate in aqueous medium at 37°C. This lability may have accounted for the observation that only microfilariae in close proximity to platelets and not those in free suspension inhibited platelet aggregability. The soluble microfilarial-derived products exhibited a dose-dependent capacity to elevate platelet cAMP (but not cGMP), which with large numbers of parasites approached levels of platelet cAMP induced by iloprost, an approximately equipotent analogue of natural prostacyclin (13). These results indicate that microfilariae may inhibit platelet aggregation by releasing factors which cause a rise in platelet cAMP.

Several naturally occurring low-molecular weight compounds are known to inhibit platelet aggregation by receptor or non-receptor-mediated stimulation of platelet adenylate cyclase. These agents include the β-adrenergic catecholamines, adenosine, forskolin, and the anti-aggregatory prostanoids, particularly prostacyclin (14). However, except for prostacyclin, all of these compounds are relatively stable and accumulate in aqueous media, and thus are unlikely to account for the observed potent but labile parasite-derived effects on platelets. In particular, adenosine, though widely distributed in nature, is not a potent inhibitor of aggregation, and its effects can be blocked by methylxanthines, which act as adenosine receptor antagonists (15). In our studies, the addition of either 3-isobutyl-1-methylxanthine or adenosine deaminase (which would destroy soluble adenosine) did not affect the capacity of microfilariae to suppress platelet responses, thus effectively excluding a role for adenosine in these observations. Another class of unstable natural compounds which can inhibit platelet function are organic nitrates, including nitric oxide (endothelium-dependent relaxing factor) or nitrosothiol compounds, which act by stimulating soluble guanylate cyclase (16). However, in our experiments, platelet levels of cGMP were not increased after exposure to the parasites, and pretreatment of the parasites with competitive inhibitors of nitric oxide formation did not affect the capacity of microfilariae to inhibit platelet aggregation.

The anti-aggregatory prostanoids are well-described and important physiological factors which inhibit platelet aggregation. These include prostacyclin, PGD_2, and PGE_2 in decreasing order of potency (17). We have previously demonstrated that microfilariae of *B. malayi* elaborate prostacyclin and PGE_2 (but not the pro-aggregatory compound thromboxane A_2) from endogenous as well as exogenous arachidonic acid (6). Release of prostanoids into the microenvironment surrounding *B. malayi* and *Wuchereria bancrofti* microfilariae can also be demonstrated by immunocytochemistry (17a). To examine a possible association between parasite-derived prostanoids and the observed effects on platelets, microfilariae were first treated with either (*a*) aspirin, which irreversibly inactivates cyclooxygenase; (*b*) indomethacin, an inhibitor of prostanoid formation whose mechanism is poorly understood; (*c*) BW755c, a pyrazoline anti-oxidant inhibitor of fatty acid oxygenation; or (*d*) ETYA, a nonmetabolizable fatty acid analogue that competitively inhibits fatty acid metabolism. The effectiveness of these agents in blocking parasite prostanoid formation was verified biochemically using radiolabeled arachidonic acid. Treatment of similar numbers of microfilariae with aspirin, indomethacin, BW755c, or ETYA resulted in a loss of the capacity of these parasites to inhibit platelet aggregation when compared to normal, untreated microfilariae. These findings indicate that microfilarial elaboration of prostanoids contributes to the inhibition of platelet aggregation.

Our results also suggest that prostacyclin is the predominant parasite-derived prostanoid involved in inhibition of platelet aggregation. Prostacyclin, the most potent known natural inhibitor of platelet aggregation (14, 15, 18), is the most abun-

dant prostanoid formed by microfilariae (6). Furthermore, it is the only anti-aggregatory prostanoid which is rapidly and spontaneously hydrolyzed within minutes (18) in aqueous media to an inactive metabolite, namely 6-keto-$PGF_{1\alpha}$. Parasite-derived prostacyclin could thus represent the bulk of the inhibitory activity, which degraded rapidly in transferred media. Because PGE_2 is a relatively weak inhibitor of platelet aggregation, it probably did not account for the small residual inhibitory activity which did not degrade rapidly in media. Transcellular metabolism of arachidonate may occur between different cell types; in particular, both endothelial cells and lymphocytes have been shown to synthesize prostacyclin from platelet-derived prostaglandin endoperoxides (19, 20). Thus, platelets could have donated prostaglandin endoperoxides to microfilariae towards the generation of prostacyclin. However, this is unlikely because similar platelet inhibitory responses were obtained using platelets treated with aspirin to block prostaglandin endoperoxide formation or using incubation media from parasites alone; furthermore, no radiolabeled prostacyclin was detected after the incubation of microfilariae with platelets prelabeled with [^{14}C]arachidonic acid (not shown). Among the eicosanoids formed by lipoxygenase enzymes, 13-hydroxyoctadecadienoic acid (derived from linoleic acid), has been postulated to inhibit platelet adhesion (21). However, in independent experiments, we were unable to detect the formation of radiolabeled 13-hydroxyoctadecadienoic acid from [^{14}C]linoleic acid by *B. malayi* microfilariae using reverse HPLC for lipoxygenase metabolites (not shown).

Proteins or other stable factors may have contributed to the residual inhibitory activity against platelets that did not degrade rapidly in media. In this regard, Foster et al. recently showed that water-soluble extracts of *B. malayi* microfilariae at a concentration of 0.1 mg/ml inhibited platelet aggregation induced by collagen and ADP, and that induced by arachidonic acid at a concentration of 0.6 mg/ml of extract (22). Microfilarial extracts as well as parasite culture medium also inhibited activation of factor XII (Hageman factor) in their study. Although the water-soluble extracts were not further characterized, these findings suggested the action of stable protein-like factors. A number of secreted parasite proteins are in fact enzymes, whose functional significance in most cases has yet to be determined (23). In terms of anti-hemostatic mechanisms, such products might not necessarily affect platelets directly but instead inhibit platelet aggregation by degrading pro-aggregatory molecules such as ADP (i.e., an apyrase-like activity), thrombin (a hirudin-like activity) or PAF. Indeed, secreted proteins from the rat nematode *Nippostrongylus brasiliensis* have recently been demonstrated to specifically inhibit PAF-induced platelet aggregation via enzymatic cleavage of PAF (24). In contrast, our results indicate a major direct effect of parasite-derived mediators on platelet responsiveness, and we did not, under the experimental conditions of this study, detect an inhibition of either thrombin, ADP, or PAF per se used as agonists.

Our results demonstrate that a helminthic parasite can actively inhibit platelet aggregation, and suggest that parasite-derived eicosanoids may down-regulate host cellular responses. Although the present study did not address the effects of microfilariae on the vascular wall, prostacyclin and PGE_2 are also potent vasodilators (25). The parasite release of these anti-aggregatory and vasodilatory prostanoids might therefore permit microfilariae to pass through capillary vessels. In the case of these bloodborne worms, such unrestricted circulation through the bloodstream is essential to sustain their parasitic life cycle. Microfilariae must be available for ingestion in the bloodmeal of vector mosquitos to reach subsequent developmental stages within mosquitos and perpetuate transmission of this parasitic infection. Because prostanoids are not stable circulating hormones (26, 27), parasite-derived prostanoids would be unlikely to exert deleterious systemic effects on the host. Indeed, bleeding phenomena due to abnormal platelet function or hemostasis is not a recognized clinical problem in microfilaremic patients. Our findings may also be germane to the intravascular stages of other metazoan parasites, including microfilariae of *Wuchereria bancrofti, Onchocerca volvulus, Loa loa,* and *Mansonella perstans,* and adult stages of the trematode *Schistosoma.* Platelets, in concert with specific antibodies, have been shown to have a cytotoxic effect in vitro against certain helminthic parasites (28–30). The parasite suppression of platelet responses demonstrated in this study may serve to counteract such potential host defense mechanisms in vivo. Helminthic parasites likely utilize diverse mechanisms, both conventional and unique, to limit platelet responses and evade host hemostasis (31). The filarial release of platelet-suppressive prostanoids may exemplify a previously unappreciated mechanism whereby eicosanoids elaborated by multicellular parasites can contribute to prolonged parasite survival within the infected human host.

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