Blood Platelets and Schistosome Egg Excretion (42993)

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Abstract. **The eggs of helminths of the Schistosoma genus require to be extravasated in order to continue the life cycle of the parasite. The possible mode by which this takes place was investigated in a mouse model. Suppression of platelet activity in *Schistosoma mansoni*-infected mice by administering rabbit anti-mouse platelet serum or a selection of "antiplatelet drugs" resulted in a significant reduction of parasite egg excretion. This reduction was best achieved when antiplatelet agents were administered just before the onset of parasite egg excretion. The association between parasite eggs and platelets was illustrated *in vivo* and *in vitro* where platelet aggregates on egg surfaces were seen in both light and electron microscopy. In addition, eggs that had been isolated from infected mouse tissues induced platelet aggregation in whole mouse blood, and this was inhibited by preincubation with the β-lactam antibiotic, ticarcillin. Isolated eggs were also capable of inducing *ex vivo* platelet aggregation in mice, which was dependent on presensitization with eggs. These data suggest a role for platelets in the extravasation and excretion of parasite eggs in schistosomiasis.** [P.S.E.B.M. 1990, Vol 193]

Although most adult helminths inhabit the lumen of the gastrointestinal tract of their respective host, those of the Schistosoma genus are unique in that they reside intravascularly. As a result, their eggs are not directly accessible to the gut or bladder lumen for excretion and subsequent continuation of the parasites's life cycle in the intermediate snail host. The eggs that remain in host tissue are known to have granulomas form around them (1), but an egg usually requires a period of approximately 3–5 days after being produced by the adult female worm for the miracidium to mature and start secreting antigenic material (2). This period of delay suggests that immature eggs might remain in the intravascular space and there is evidence to suggest that soon after production by the female worm, schistosome eggs are entrapped in platelet-rich thrombi (3) in a manner similar to that described for disseminating tumor cells (4–6). Since platelet-tumor cell interactions have been associated with establishment of secondary tumors (7–9), it was proposed that platelets might have a similar role in the extravasation of intravascular schistosome eggs. This was investigated by treating *S. mansoni*-infected mice with antiplatelet serum and drugs, both of which independently induced significant reductions in the rate of fecal egg excretion. In addition, evidence of a direct interaction between platelets and eggs was obtained from *in vivo*, *in vitro*, and *ex vivo* experiments.

Materials and Methods

Animals and Infections. Eight- to 10-week-old, inbred CBA strain (CBA/Ca or CBA/H-T6T6) mice were percutaneously infected with *S. mansoni* cercariae according to the method described by Smithers and Terry (10). Outbred T.O. strain mice were used as a source of mouse blood for the *in vitro* platelet assays. Worms were extracted from freshly killed mice by perfusion (10).

Fecal Egg Counting. The rate of fecal egg excretion was determined by use of the method described previously (11). Briefly, fecal pellets (10–40 mg) were collected from individual mice on the days required, before and after treatment. The pellets were weighed, disrupted in isotonic saline, and the suspension allowed to filter through a No. 4 Whatman filter paper (Whatman Ltd.) with the assistance of a suction pump. The eggs and fecal material remaining on the filter paper were stained with saturated ninhydrin solution and then allowed to dry at 37°C. The number of eggs on each filter paper was counted at a magnification of ×32 and expressed as the number of eggs/100 mg of fecal matter.

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Tissue Egg Counts. The tissue egg load was determined by using a previously described method (11). Briefly, after perfusion of mice for worms, the intestines and liver were extracted from each mouse and then stored at −20°C until required. The liver and intestines from each mouse were digested separately in 20 ml of 5% potassium hydroxide solution and left overnight at 37°C. The number of eggs in 50 μl of the digests was determined microscopically (×32 magnification). From this value, the number of eggs/worm pair was determined after counting the worms from the perfusate of each mouse.

Electron Microscopy. Livers and intestines were extracted from mice after 60 days of infection with *S. mansoni* and cut into 1- × 1-mm blocks which were then immediately fixed in glutaraldehyde. Tissues were prepared and sectioned for electron microscopy as described elsewhere (12).

Granuloma Measurements. The two superior lobes of the liver from each mouse were fixed in formal saline and embedded in wax for histopathology in which sections (5-μm thin) were cut and subsequently stained with hematoxylin and eosin (13). The areas of the granulomas were measured by use of a computer-controlled microscope. At least 20 granulomas/slide were measured and the mean of each sample of granulomas used as the measurement for an individual mouse.

Egg Isolation. *S. mansoni* eggs were isolated by using the method described by Doenhoff *et al.* (15). Briefly, livers and intestines from heavily infected mice that had been treated with 0.125 mg/mouse of hydrocortisone acetate were homogenized in 750 ml of isotonic saline by using a vortex blender. The resultant homogenate was digested with 1 g of pancreatic trypsin (trypsin Type 2; Sigma) for 2 hr to allow the separation of eggs from the fibrous tissue within the granulomas. Eggs were then separated from the homogenate by repeated filtrations through 180- and 300-μm brass mesh sieves and, subsequently, by repeated gravity sedimentations and 1-min centrifugations at 180*g*. Eggs with minimal mouse tissue contamination were separated, counted, and stored in the 1.8% NaCl solution at 4°C in preparation for *in vitro* assays on the following day.

Platelet Aggregation Measurement. Platelet aggregation in whole blood was measured by use of a modified version of the method described by Heptinstall *et al.* (16). Briefly, a fixed volume (10%, v/v) of a suspension of washed eggs was added to a sample of whole blood, already incubated at 37°C for 2 min, in a Payton platelet aggregometer (Payton Inc.). Platelet aggregation was denoted by a drop in platelet count (after 3 min) from that of a comparable blood sample to which saline alone was added. Results were expressed as a percentage reduction in platelet count.

Preparation of Antiplatelet Serum. Antiplatelet serum was raised in rabbits according to the method described by Gasic *et al.* (17). Briefly, platelet-rich plasma was obtained from fresh mouse blood by centrifugation at 160*g* for 10 min. Platelets were then pelleted and washed five times in Tyrode's buffer (pH 6.8) and finally suspended in phosphate-buffered saline (pH 7.4). The suspension was then injected into a New Zealand rabbit (5 ml of 20 × 10^9 platelets/ml). Two intravenous injections were given over 14 days and the rabbit was exsanguinated 7 days after the last injection. The serum was absorbed with washed mouse red blood cells for 4 hr and then decomplemented (at 56°C for 30 min). Mice which were subcutaneously injected with 100 μl of serum suffered a >95% thrombocytopenia after 24 hr. Hematocrits and white blood cells were only marginally (insignificantly) reduced.

Drugs. ϵ-Aminocaproic acid was obtained from Sigma. Ticlopidine, anegrilide and sulfinpyrazone were gifts from Dr. Barbara Nunn of Beecham's Pharmaceuticals. Ticarcillin and flucloxacillin were gifts from Dr. Soulal also of Beecham's Pharmaceuticals.

Statistical Analysis. Numerical results were expressed as the group mean ± 1 SD. The degree of significance between two mean values was determined by use of Student's *t* test and a *P* value of 0.05 or less was regarded as statistically significant.

Results

Evidence for the *in vivo* association between *S. mansoni* eggs and platelets was illustrated in both light (Fig. 1a–c) and electron microscopy (Fig. 1d). Mice that were infected with *S. mansoni* (150 cercariae/mouse) were killed after the onset of egg laying and the tissues examined microscopically. In Figure 1a a section of small intestine can be seen with eggs which are still intravascularly situated and with adherent platelet aggregates. In Figure 1b a higher magnification of a section taken from liver tissue shows intravascular eggs seemingly bound to each other predominantly by platelet aggregates. A similar association between platelets and schistosome eggs was also shown *in vitro* in which freshly isolated schistosome eggs were added to citrated whole blood from uninfected mice, and cellular aggregates, made up predominantly of platelets, were formed on the eggs' spine and on either pole (Fig. 1c). Part of the material on either pole was secreted from the egg in early hatching. The platelets seen on the spine seem to have formed an independent focus of aggregation. Electron microscopy of the *in vivo* association between platelets and schistosome eggs showed flattened, activated platelets (with partial degranulation) on the surface of an egg located in a venule of the small intestine (Fig. 1d).

In order to determine whether the association between eggs and platelets had any role in the extravasation of eggs, *S. mansoni*-infected mice were injected with rabbit anti-mouse platelet serum (APS). This re-

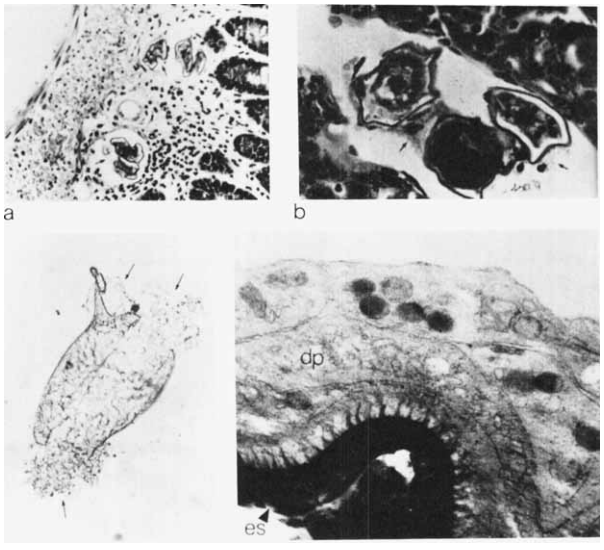

Figure 1. (a) Light micrograph of the intestinal wall of a *S. mansoni*-infected mouse. Eggs inside terminal venules with adherent platelet aggregates (In the light micrographs of *in vivo* material, sections were also stained with Picromallory stain in order to identify platelets (stained blue).) (arrows). Some eggs adherent to each other and onto vascular endothelium (5 μm, hematoxylin and eosin; original magnification ×160). (b) Three eggs inside hepatic vasculature of an infected mouse showing large platelet aggregates (In the light micrographs of *in vivo* material, sections were also stained with Picromallory stain in order to identify platelets (stained blue).) (arrows) and few mononuclear leukocytes linking the eggs (5 μm, hematoxylin and eosin; original magnification ×400). (c) *S. mansoni* egg taken from an *in vitro* reaction of platelets (in whole mouse blood) with eggs. Platelet aggregates concentrated around egg's spine and at sites of hatching (both indicated by arrows original magnification ×400). (d) Electron micrograph of intravascular egg (es) with activated platelets on its surface. Activation indicated by degranulation (dp) in one the the platelets (original magnification ×32,000).

sulted in significant reductions of fecal egg excretion rates (Table I). APS administration after the onset of egg laying (which occurs approximately 30 days after infection in this model) at 35 days postinfection resulted in a significant reduction of egg excretion. This reduction was further enhanced when the APS was administered (to a separate age/sex-matched controlled group of mice) 42 days after infection (Table I), although this group suffered a high mortality rate (67%). Corresponding tissue egg burdens and adult worm numbers indicated that the egg-laying capacity was not compromised by APS treatment (Table I). The high mortality rate associated by APS treatment was circumvented by administering drugs (instead of serum) with known antiplatelet activity. Significant reduction in fecal egg numbers (ranging from 31 to 81%) was observed in infected mice treated with sulphinpyrazone, ticlopidine, anegrilide, and the β-lactam antibiotics, ticarcillin and flucloxacillin (Table I). Treatment with ϵ-aminocaproic acid, an antifibrinolytic drug with no known antiplatelet activity, did not, however, result in a reduction of fecal egg numbers. The lack of any significant differences in the tissue egg loads of the mice treated with antiplatelet drugs implies that the drugs did not affect the rate of egg production.

In vivo evidence of the association between eggs and platelets was demonstrated when the circulating platelet count was raised after *S. mansoni*-infected mice (rendered thrombocytopenic by an egg-laying infection) had been treated with a drug with potent antiplatelet activity in mice, i.e., ticarcillin (18) (Table II). The *in vivo* efficacy of the drug was further illustrated by the

Table I. Effect of Antiplatelet Agents on Fecal Egg Excretion[a]

Experiment	Drug	No. of mice (% mortality)	Fecal egg no. (% reduction)	Tissue eggs ($\times 10^3$)	Worm counts
1	Control	6	77 ± 90	56.7 ± 0.95	62 ± 6
	APS (day 32)	10	22 ± 24 (71%)*	55.5 ± 23.13	62 ± 16
	APS (day 42)	3 (70%)	2 ± 4 (97%)***	—	—
2	Control	5	138 ± 72	85.1 ± 23.3	84 ± 34
	Sulfinpyrazone	8	95 ± 83 (31)*	72.1 ± 40.9	84 ± 38
	Anegrilide	7 (12.5%)	40 ± 36 (71)**	63.3 ± 19.6	74 ± 19
	Flucloxacillin	7 (12.5%)	48 ± 46 (65)*	68.8 ± 16.4	75 ± 14
3	Control	7	637 ± 230	41.2 ± 15.2	44 ± 6
	Ticarcillin	7 (12.5%)	124 ± 97 (81)***	46.5 ± 17.0	40 ± 14
4	Control	6	90 ± 32	14.1 ± 6.9	75 ± 22
	Ticlopidine	3 (62.5%)	25 ± 22 (72)*	14.5 ± 6.0	67 ± 14
5	Control	5	116 ± 37	ND	77 ± 21
	EACA	8	149 ± 62 (−28)	ND	79 ± 29

[a] *S. mansoni*-infected mice (150–200 cercariae/mouse) were treated with antiplatelet drugs starting from Day 38 of infection. Fecal pellets were collected daily starting from Day 42 until Days 46 and 47. Mice were perfused for adult worms, and liver and gut tissues were extracted for later estimation of the tissue egg loads (see Materials and Methods). Heterologous antiplatelet serum was given as a single subcutaneous injection (0.025 ml/mouse) on Day 35 or Day 42 (depending on the group). Platelet numbers were reduced by >95% of the control count for approximately 48 hr after APS injection and rose to the control count by the fourth day. The value of the fecal egg numbers refers to the number of eggs in each 100 mg of fecal matter. Drugs used were sulfinpyrazone (120 mg/kg orally, three times a day), anegrilide (23 mg/kg orally, once daily), flucloxacillin (320 mg/kg, three times a day, Days 38 and 39 only), ticlopidine (300 mg/kg orally, three times a day), and ϵ-aminocaproic acid (10 mg/kg subcutaneously, 4 times a day). The percentage of mortality rates is indicated (in parentheses) only in the groups in which mice died. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$. Numbers in parentheses (in the second row of table) denote the percentage of reduction of fecal egg numbers. ND, not done.

marginally higher egg numbers in the guts of treated mice, although granuloma sizes were unaltered (Table II). The higher tissue egg numbers/worm pair suggest a build-up of unexcreted eggs.

In order to confirm the antiplatelet activity of ticarcillin on egg-induced platelet aggregation, the efficacy of the drug was also tested *in vitro*. Thus, ticarcillin was preincubated with whole mouse blood before addition of freshly isolated eggs, and this resulted in a reduction of platelet aggregation of 74% (16 ± 7%, drug-treated, vs 62 ± 22%, control group, $P < 0.001$). Injections of isolated eggs into uninfected, unsensitized mice (via the tail vein) also rendered the mice thrombocytopenic (40–50% after 2 hr, fig 2a). In comparison, egg-sensitized mice which were challenged intravenously with freshly isolated schistosome eggs suffered a comparable decline in circulating platelet counts after only 5 min (Fig. 2b), followed by a rapid but partial

Table II. Effect of Ticarcillin on Platelet Function in *S. mansoni*-Infected Mice[a]

	Control ($n = 6$)	Ticarcillin ($n = 8$)
Circulating platelets ($\times 10^8$/ml)	5.45 ± 0.22	7.86 ± 0.92
Tissue eggs/worm pair		
Gut	1779 ± 388	2436 ± 699
Liver	428 ± 147	409 ± 134
Granuloma area (mm^2)	10.22 ± 1.68	10.44 ± 1.67

[a] Ticarcillin (320 mg/kg, three to four times a day) was administered to *S. mansoni*-infected mice (150 cercariae/mouse) on Days 39 and 40 after infection. Platelets were counted on 4 separate days before perfusion and, similarly, fecal eggs were counted as described previously. Platelets were counted daily after onset of drug treatment and the maximum counts (after 2 days) are indicated. All mice were perfused for adult worms on Day 47. The superior two lobes of the livers were collected and fixed in formal-saline and stained with hematoxylin and eosin for measurement of granuloma sizes. The remainder of the liver and intestine was digested for estimation of tissue egg numbers.

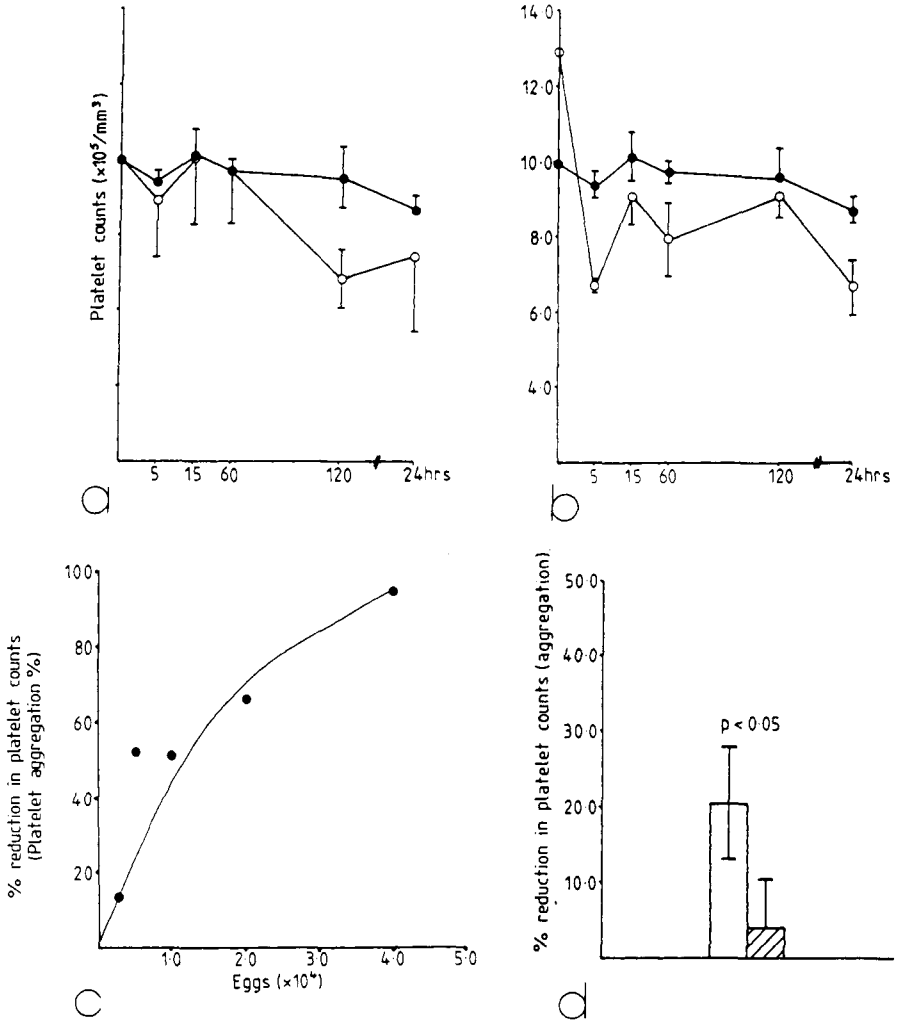

Figure 2. Egg-induced platelet aggregation *ex vivo* and *in vitro*. (a) Naive (unsensitized) mice were injected with 2000 eggs via the tail vein and platelets counted at specified intervals. These platelet counts were compared with those of the uninjected matched control group of mice. (b) Mice were challenged with eggs via the tail vein (2000 egg/mouse) having been sensitized 4 weeks earlier with 10,000 eggs each (intraperitoneally). Mice were bled and platelets counted at specified intervals after the challenge egg injection (*ex vivo* platelet aggregation). (c) *In vitro* platelet aggregation in whole blood induced by freshly isolated *S. mansoni* eggs (dose-response curve, 0.25–4.0 × 10^4 eggs/ml). (d) Egg-induced platelet aggregation in citrated (3.2 mg of trisodium citrate/ml of blood) whole mouse blood: effect of preincubation of blood with serum from chronically infected mice (1:20) (open symbol) and normal mouse serum (hatched symbol); eggs, 0.4 × 10^4 egg/ml (1 part of egg suspension to 10 parts of blood).

reversal in circulating platelet count. Egg-induced platelet aggregation was also measured *in vitro* in whole blood and occurred in a dose-dependent, saturable manner (Fig. 2c). From the same samples of blood, sedimented eggs with platelet aggregates on their surfaces were viewed microscopically (Fig. 1c). In agreement with the *ex vivo* results, preincubation of whole mouse blood with serum from chronically infected mice (rich in anti-egg antibodies) resulted in an enhancement of platelet aggregation, depicted as the percentage of reduction in platelet numbers (Fig. 2d). A low concentration of washed eggs (0.4×10^4/ml in 1.8% NaCl solution) which induced minimal platelet aggregation in platelet-rich plasma preincubated with normal mouse serum induced a response which was four times as marked when platelet-rich plasma was incubated with serum from chronically injected mice.

Discussion

By using several different antiplatelet agents in *S. mansoni*-infected mice, it was possible to conclude that platelets or factors associated directly with platelet function are responsible for assisting the extrusion of eggs from the intravascular compartment. The antiplatelet agents used here are likely to be active against other host cells in addition to platelets. In the case of APS, sera raised thus against platelets are likely to show cross-reactivity at least with endothelial cells (20). Thus, part of the effect induced by APS may be due to its effect on endothelial cells and this might explain the high mortality rate in infected mice given APS after the onset of egg excretion (Day 42). An already delicate endothelium subjected to a large egg load being extravasated may be unable to control the extensive bleeding which results after APS is administered. However, in spite of the different modes of action, on platelet function, of sulfinpyrazone (21), anegrilide (22), ticlopidine (23, 24), and β-lactam antibiotics (25), each of these drugs was independently capable of suppressing schistosome egg excretion. The consistency with which egg excretion was inhibited by these drugs, therefore, strongly suggests that compromised platelet activity was responsible for inhibiting egg excretion.

In view of the results indicating the reversible manner with which platelets interact with eggs in sensitized mice (Fig. 2b), it may be assumed that egg-induced platelet aggregation is reversible and, as a result, may not induce substantial fibrin formation. Indeed, in previous work (19), mice infected with *S. mansoni* suffered a thrombocytopenia which was first noticed around the time that egg laying commenced. Although there is evidence to show that this reduction in platelets is immune dependent (Ngaiza *et al.*, submitted for publication), there is a suggestion from these results that at least part of this thrombocytopenia is induced by eggs. Platelets aggregate on the egg surface but in the presence of immune serum this aggregation is enhanced and reversible (Fig. 2b). This enhancement in platelet activity may have been due to the formation of antigen/antibody complexes around the challenge eggs (26) which would have then activated platelets via the Fc receptor. The primary egg component responsible for aggregating platelets may thus act in conjunction with newly formed antigen/antibody complexes around eggs in producing a stronger platelet response. In addition, antibodies against platelet antigens which are present in serum of *S. mansoni*-infected mice (Ngaiza *et al.*, submitted for publication) may also play a role in modulating the platelet response to eggs. In this way, platelets may be able to assist in the extravasation of eggs while at the same time avoiding thrombosis. Platelets may also promote the extravasation of eggs by releasing products such as platelet factor 4, an immune stimulator (27), or eicosanoids which in turn activate leukocytes such as neutrophils (28).

The data presented in this report also show many similarities to the relationship between platelets and tumor cell metastasis (6–8). Although the exact role of platelets in tumor metastasis has yet to be clarified, a proposed sequence of events may be as follows: most disseminating tumor cells are destroyed intravascularly (29) and the few that survive may induce platelets to aggregate around them, thereby protecting them from complement, natural killer cells, cytotoxic T cell activity, or other possible cytotoxic processes in plasma (30). Embolized tumor cells subsequently lodge onto the vascular endothelium with the help of activated platelets on the surface of the emboli (4) and, through mechanical and proteolytic action of the tumor cells, are eventually extruded into the extravascular space.

Schistosome eggs may follow a similar sequence of events and, as a result, exploit platelet activity in order to ensure their continued survival. This parallel between tumor metastasis and schistosome egg excretion suggests that a similar mechanism may apply to the survival of other microbial and parasitic pathogens within the vasculature of their mammalian hosts. Indeed, in tuberculosis, another granuloma-producing infection, a direct association between newly extravasated tubercle bacilli with platelet aggregates has been reported (31).

Entrapment of microbial organisms within platelet aggregates, as is often demonstrated in both *in vitro* and *in vivo* studies involving the use of bacteria or viruses (32–36), might have evolved primarily for the purpose of host defense. In the case of mice (and possibly other permissive hosts) infected with *S. mansoni*, it can further be suggested that the evolutionary process of the parasite may have advanced further than that of the host so as to not only withstand the host's hostile environment but to also make use of the latter as a requirement for its own continued survival. It may be that platelets only participate in this process by providing receptors for the Arg-Gly-Asp tripeptide, which is

present in most adhesive proteins such as fibronectin, laminin, fibrinogen, and vitronectin (37). These receptors have been specifically associated with the GpIIb-IIIa complex on activated platelets in tumor metastasis in mice (38). Indeed, the central role of this heterodimer in tumor growth and metastasis was recently demonstrated in a melanoma cell line in which pretreatment of the tumor cells *in vitro* with a monoclonal antibody against the GpIIb-IIIa complex resulted in complete suppression of growth of the tumor in mice (39). The GpIIb-IIIa complex might then bind to any of a number of adhesive proteins (mentioned above), one or some of which are accessible on the activated endothelial surface (4). Platelet activation may therefore be such that significant platelet release of procoagulants does not occur (or is rapidly neutralized). This suggests that the parasite is able to evade the possible harmful effects which are often induced by platelet aggregation while at the same time selecting the form of platelet activity which will be to its benefit.

Although many similarities between tumor cells and schistosome eggs are evident, schistosome eggs, unlike disseminating tumors, are organisms (miracidia) covered by a tough chitinous shell and do not self-replicate. However, despite the lack of new sites of egg production, the continuous production of eggs by female worms whose natural habitat is the blood circulatory system of the mammalian host makes up for this apparent deficiency in the parasite's life cycle. Tumor cells implanted at tissue sites distant from the primary tumor form secondary tumors, and their growth is known to receive support from an equally rapidly growing blood supply through the production of angiogenic factors by the tumor cells (41). These are necessary for the formation of new blood vessels which are crucial to the survival of many different malignant tumors (41). A similar factor in *S. mansoni* eggs was recently reported (42) and, it is assumed, has a similarly critical role in supporting the growth of tissue granulomas which in themselves play an important role in parasite egg excretion (3, 43).

The process of schistosome egg excretion has also been shown to depend on both humoral and cellular aspects of immunity (3, 43). The granuloma, the formation of which is immune dependent (1), was shown both in this laboratory (3) and in that of Damian's (43) to play a central role in the excretion of schistosome eggs. As postulated by Damian (43), the tissue granuloma may serve as a vehicle for transporting eggs through the intestinal wall and into the lumen. This would presumably only be possible after extravasation of eggs.

Unraveling of the possible mode by which platelets and the immune response may relate to each other in facilitating the extravasation and excretion of schistosome eggs could improve our understanding of the host-parasite relationship of this and other similar infections. In order to elucidate this relationship, detailed biochemical and immunologic analysis of the association between platelet membrane and schistosome egg surface antigens will be required.

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