Schistosoma mansoni Soluble Egg Antigens Trigger Erythrocyte Cell Death

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Key Words

Schistosomiasis • Anemia • SEA • Cell death • Calcium

Abstract

Estimated to affect nearly 300 million people worldwide, schistosomiasis is caused by parasitic flatworms of the genus *Schistosoma*. The major pathological consequences of chronic schistosomiasis are associated with soluble egg antigens (SEA) secreted from schistosome egg deposits in liver and other organs. The vigorous immune responses induced by egg antigens result in granuloma formation and other pathophysiological symptoms such as hepatosplenomegaly and anemia. Risk of anemia correlates with infection intensity and the level of host hemoglobin is inversely proportional to egg count. Schistosomiasis-associated anemia could be multifactorial, but the potential link and molecular underpinnings are unclear. Here, we evaluate whether *S. mansoni* SEA affects survival of mouse erythrocytes. Erythrocytes incubated with different concentrations of SEA were tested for various markers of erythrocyte cell death. Erythrocytes exposed to SEA exhibit elevated intracellular Ca^{2+} levels as

measured by Fluo-3 AM fluorescence in flow cytometry, and they also display concentration-dependent, Ca^{2+} -dependent, and heat-sensitive increases in phosphatidyl serine exposure. Further, SEA-exposed erythrocytes show increased fluorescence using the *in situ* apoptosis marker CaspACE FITC, indicating the involvement of caspase-mediated cell deformation. Taken together, these results offer several lines of experimental evidence for SEA-induced erythrocyte cell death and may provide new insights into factors contributing to schistosomiasis-associated anemia.

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Introduction

Schistosomiasis is a leading tropical disease caused by flatworms of the genus *Schistosoma*. It is estimated to affect over 200 million people globally and cause nearly 300,000 deaths annually [1, 2]. The sexually dimorphic adult worms reside as pairs in the host mesenteric veins (*S. mansoni*, *S. japonicum*) or venous plexus of the bladder (*S. haematobium*) and lay eggs at average rates ranging from 300 - 3000 eggs/day/worm. Eggs which are

not excreted into feces (or urine) to continue the parasite life cycle remain in the host and evoke a host immunopathological response, resulting in the major pathophysiological consequences associated with schistosomiasis. Schistosomiasis has a major impact on global health, as measured by estimated disability-adjusted life years (DALYs) lost annually [3], and severe chronic infections can result in pulmonary granuloma/fibrosis formation, portal hypertension, non-functioning kidney, hepatosplenomegaly, and subsequent death.

Schistosomiasis-associated anemia is well documented [4-7] and recent evidence evaluating effects of reinfection indicate a causal relationship [8]. Anemia, defined by the World Health Organization as a reduction in hemoglobin to levels below 11 - 13 gm/dl, is a significant public health burden in developing countries, and if left untreated, can lead to numerous complications including cognitive impairment and nerve damage [9]. Several factors have been proposed to underlie schistosomiasis-induced anemia [4]. These include elevated levels of pro-inflammatory cytokines, such as tumor necrosis factor α (TNF- α) and interleukin-6 (IL-6), resulting in anemia of inflammation; autoimmune hemolysis; sequestration and hemolysis of red blood cells; and iron deficiency via urinary and fecal blood loss. However, the molecular mechanisms involved in mediating this pathology are not clear, and understanding has been hampered by symptomatic heterogeneity and presence of concomitant, multiple infections [10, 11]. Given the fact that eggs, but not worms, serve as the major contributor of morbidity associated with schistosomiasis [12], we have evaluated whether schistosome eggs may be implicated in fostering anemia directly, by measuring the effect of soluble egg antigen (SEA) from *S. mansoni* on survival of mouse erythrocytes.

Like nucleated cells, erythrocytes can also undergo cell death, or eryptosis [13-16], through the activation of Ca^{2+} -permeable non-selective cation channels by multiple factors, such as oxidative stress, osmotic shock [17], energy depletion [18], PGE_2 [19], and infection [20, 21]. The resultant Ca^{2+} influx elicits an apoptotic-like signaling cascade, with effects including cell shrinkage through modulation of Ca^{2+} -dependent K^+ channels (with KCl co-transport) [22]. Ca^{2+} can also act as a secondary messenger in activation of scramblase and calpain, resulting in breakdown of membrane phospholipid asymmetry and cell deformation [19]. Phosphatidyl serine (PS) exposure is a hallmark of apoptosis (and eryptosis), typically leading to cells being engulfed and eliminated from the circulation by macrophages endowed with

phagocytic receptors for PS [23, 24]. Accordingly, inhibition of the erythrocyte cation channel abolishes Ca^{2+} -induced erythrocyte death [25, 26].

Infection with schistosomes has been shown to induce apoptosis in a variety of immune cells, such as splenocytes and CD4^+ cells. In particular, SEA mediates the cell death of CD4^+ T cells through the binding of Fas ligand [27]. Egg excretory/secretory products also alter the Fas-Fas ligand system, a major apoptotic pathway, in mouse liver [28]. In the present study, we investigated the effect of SEA on mouse erythrocytes. We show that exposure of erythrocytes to SEA induces several markers of eryptosis in a concentration-dependent manner. We interpret these results to define a previously unrecognized factor that likely contributes to the development of schistosome-associated anemia.

Materials and Methods

Collection of erythrocytes

Erythrocyte samples were collected from 6-8 week old Swiss webster mice (Charles River Laboratories). Cells were washed in a Ringers solution containing 125 mM NaCl, 5 mM KCl, 1 mM MgSO_4 , 5 mM glucose, 1 mM CaCl_2 , 32 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (pH 7.4) after removing plasma and buffy coat by centrifuging at $1500 \times g_{\text{max}}$ for 2 min. Where indicated, erythrocytes were incubated in Ringers solution in the presence or absence of Ca^{2+} ($\pm$ 5mM EGTA), and with or without SEA (50-200 $\mu\text{g/ml}$) for 48 h at 37°C .

Preparation of S. mansoni soluble egg antigens (SEA)

SEA was prepared as described previously [29]. Briefly, livers were isolated from mice 7-8 weeks post infection, tissues were minced with scissors in 1.2% NaCl and passed through a crude sieve. The filtrate was passed through a series of sieves with decreasing pore size and finally eggs were collected from the top sieve (45 μm). To collect the mature eggs, a repeated swirling technique was employed and eggs were washed with several volumes of 1.2% NaCl solution. After purification, eggs were suspended in ice-cold PBS containing protease inhibitor cocktail (Sigma) and homogenized with a polytron homogenizer (PT1200 E) at 4°C . Following ultracentrifugation at $100,000 \times g_{\text{max}}$ for 1 h at 4°C , the crude supernatant was sterilized by passing through a 0.2 μm filter. The protein concentration was determined using a Bradford assay (Fermentas) and the final supernatant was stored at -80°C until further use. For heat treatment, SEA samples were incubated at 90°C for 10 min prior to use.

Fluorescence measurements

For Ca^{2+} levels or caspase activity, erythrocytes were loaded with 2 μM of the cell-permeant fluorescence dye fluo-3 AM (Invitrogen) or CaspACETM FITC-VAD-FMK (Promega) at a final concentration of 5 μM for 30 min at 37°C in Ringers

solution. To analyze the externalization of phosphatidyl serine, cells were incubated with Annexin V-FLUOS (Roche) at 1:500 dilution for 10 min at 37°C in the labeling solution supplied by the manufacturer. After incubation, all samples were washed twice with Ringers solution and the fluorescence intensity was analyzed in the FL-1 fluorescence channel with excitation at 485 nm, emission at 520 nm on a FACS Calibur™ instrument (BD Biosciences).

Cell size measurements

To measure cell size, erythrocyte samples were washed and re-suspended in Ringers solution. For flow cytometric analysis, cells were analyzed by forward scatter, with 15,000 events from each sample measured.

Hemolysis

After incubation of erythrocytes with SEA, the supernatant was collected by centrifugation at $1000 \times g_{\max}$ for 5 min at room temperature and the release of hemoglobin evaluated spectrophotometrically at 540 nm (Labscan Multiscan Plus). As a positive control for 100% hemolysis, erythrocytes were lysed hypotonically in water and this sample used as a standard to calculate the percentage of hemolysis.

Statistical analysis

Data were statistically analyzed in GraphPad Prism 5.03 software using either ANOVA with Tukey post-test or unpaired, 2-tailed *t*-tests. Where mentioned “n” indicates the number of times the experiment was repeated, and data are expressed as the arithmetic mean $\pm$ SEM. $P < 0.05$ was considered as significant.

Results

SEA increases free cytosolic Ca^{2+} levels in erythrocytes

Changes in levels of intracellular Ca^{2+} are associated with erythrocyte cell death [30-32]; hence, we initially analyzed erythrocytes for SEA-stimulated changes in intracellular Ca^{2+} levels, using fluo-3 AM, a cell-permeant, high affinity fluorescent Ca^{2+} indicator. As shown in Fig. 1, erythrocytes incubated with SEA for 48 hrs display a concentration-dependent increase in cytosolic Ca^{2+} levels compared to control erythrocytes.

SEA increases phosphatidyl serine (PS) exposure

Increases in cytosolic Ca^{2+} mediate several physiological responses, including the breakdown of phospholipid asymmetry by Ca^{2+} -dependent proteases [19, 31]. To test whether SEA-exposed erythrocytes also respond in this manner, erythrocytes were incubated with 50-200 $\mu\text{g/ml}$ of SEA, and the percentage of PS-exposing cells was determined by labeling with annexin-V, which

Fig. 1. Cytosolic free Ca^{2+} levels ($i[Ca^{2+}]_{\text{free}}$) increase in erythrocytes exposed to SEA. A. Flow cytometry histogram of fluorescent Ca^{2+} indicator fluo-3 in erythrocytes in the absence (control) or presence of 200 $\mu\text{g/ml}$ SEA. The intensity of fluorescence increases in erythrocytes incubated with SEA for 48 h, indicating an elevation in intracellular Ca^{2+} levels. B. Mean fluo-3 fluorescence of control erythrocytes (black bar; $\pm$ SEM; $n = 10$) or erythrocytes treated with different concentrations (50-200 $\mu\text{g/ml}$) of SEA (grey bars; $\pm$ SEM $n = 5-10$). SEA significantly increases intracellular Ca^{2+} levels in a concentration-dependent manner (* and *** indicate $P < 0.05$ and $P < 0.001$ respectively).

has a strong, specific affinity for PS, and is a marker for erythrocyte cell death [16]. As demonstrated using flow cytometry (Fig. 2), SEA significantly promotes the externalization of PS in a concentration-dependent manner, a distinct feature of programmed cell death, and an effect that correlates with the elevated Ca^{2+} activity we observe in SEA-treated erythrocytes. In contrast, control cells unexposed to SEA show only negligible amounts of PS exposure.

SEA does not alter cell size, but stimulates erythrocyte caspase activity

To determine the effect of SEA on erythrocyte size, cells were analyzed by forward scatter in flow cytometry. As shown in Fig. 3, treatment with SEA did not induce cell size reduction, indicating that erythrocyte death is

Fig. 2. SEA increases PS exposure at the erythrocyte cell surface. A. Flow cytometric analysis of Annexin-V-FLUOS labeling of erythrocytes in the absence of SEA (control) or erythrocytes treated with 200 $\mu\text{g/ml}$ SEA. Erythrocytes were incubated in Annexin-V-FLUOS labeling solution at 37°C for 10–15 min. In comparison to control erythrocytes, SEA-treated cells show a significant population of cells staining positive for annexin V, indicating higher levels of exposed PS. B. Data representing mean ($\pm$ SEM, $n = 9$ –14) percentage of annexin V labeling in cells incubated without (black bar) or with (grey bars) different concentrations of SEA (50–200 $\mu\text{g/ml}$) for 48 h at 37°C. The PS exposure on the outer leaflet of the erythrocyte cell membrane increases in a dose-dependent manner. *, **, *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, ANOVA.

independent of cell shrinkage, as is seen in other examples of erythrocyte cell death [33–35].

Erythrocytes are known to express apoptotic protease molecules, such as caspase-3, caspase-8, and calpain [14, 36]. To test for activated caspases, cells were analyzed with the *in situ* marker CaspACE™ FITC-VAD-FMK a fluorescent analog of the pan caspase inhibitor Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone). As shown in Fig. 4, SEA triggers an approximately 3-fold increase in fluorescence, but only at 200 $\mu\text{g/ml}$, and not at lower concentrations. Thus, caspases appear to be activated, though at higher concentrations than required for activation of other markers. Activated apoptotic molecules damage the plasma membrane and trigger membrane blebbing, although suicidal cell death does not lead to cell lysis, in contrast to necrosis, in which cells lyse and release their contents. Therefore, we determined the percentage of hemolysis as a measure of total cell death. As shown in Fig. 5, SEA induces a release of hemoglobin into the incubation medium, indicating damaged cell membranes and hemolysis, only at 200 $\mu\text{g/ml}$, the highest concentration tested. Lower concentrations of SEA that trigger markers of cell death do not produce significant hemolysis.

SEA-induced erythrocyte cell death requires Ca^{2+} and is heat-labile

To investigate the role of Ca^{2+} in SEA-induced erythrocyte cell death, we incubated erythrocytes in EGTA-buffered Ringers solution and analyzed PS exposure, a hallmark of eryptosis. The absence of Ca^{2+} significantly reduced PS externalization (Fig. 6), but did not abolish it completely. This result provides strong evidence for Ca^{2+} dependence of SEA-induced erythrocyte cell death.

As an initial step in characterization of the component(s) of SEA that triggers erythrocyte cell death,

Fig. 3. SEA does not alter erythrocyte cell size. A. Forward scatter histogram of control erythrocytes or erythrocytes incubated with 100 $\mu\text{g/ml}$ or 200 $\mu\text{g/ml}$ SEA for 48 h in Ringer solution at 37°C. Conditions represented by each histogram are as noted on the figure. B. Arithmetic mean ($\pm$ SEM, $n = 5$ –9) of forward scatter analysis from control (black) or SEA-treated (50–200 $\mu\text{g/ml}$) erythrocytes (grey bars) in Ringer solution for 48 h. FACS analysis of 15,000 live gated cells per sample.

Fig. 4. SEA stimulates erythrocyte caspase activity. A. Flow cytometry plot of caspase activity in control vs. SEA-treated (200 $\mu\text{g/ml}$) erythrocytes following incubation for 48 hr. The SEA treatment increases the caspase activity as detected by CasPACETM FITC fluorescence. B. Data representing percentage ($\pm$ SEM, $n = 5-8$) of caspase activity in control (black bar) or SEA-treated (50-200 $\mu\text{g/ml}$) erythrocytes (grey bars). *** indicates $P < 0.001$; ANOVA.

Fig. 5. SEA disrupts erythrocyte membrane integrity at 200 $\mu\text{g/ml}$. Mean percentage ($\pm$ SEM, $n = 10$) of hemolysis for erythrocytes incubated in the absence (black bar) or presence (grey bars) of SEA (50-200 $\mu\text{g/ml}$) in ringer solution for 48 h at 37°C. The release of hemoglobin into the incubation buffer was measured at 540 nm in a plate reader at room temperature. Only 200 $\mu\text{g/ml}$ SEA appears to significantly ($P < 0.001$, ANOVA) disrupt the erythrocyte cell membrane, leading to cell lysis.

Fig. 6. Effects of SEA on erythrocyte PS exposure are Ca^{2+} -dependent. Mean annexin V fluorescence ($\pm$ SEM, $n = 6-9$) of cells untreated (black) or treated with SEA in the presence (grey bars) or absence (white bar) of 5 mM EGTA. The absence of Ca^{2+} significantly ($p < 0.001$, ANOVA) reduces PS exposure.

we determined whether the effect is sensitive to elevated temperature. As shown in Fig. 7, SEA heated to 90°C for 10 min prior to incubation with mouse erythrocytes was inactive in promoting PS exposure as measured by annexin V labeling. Thus, the induction of erythrocyte cell death by SEA appears to be a heat-labile function.

Discussion

The scale of the global impact of schistosomiasis on human health has historically been underestimated [3]. Schistosomiasis-associated anemia represents one

Fig. 7. Stimulation of erythrocyte cell death by SEA is heat-labile. Annexin V labeling of erythrocytes (as in Fig. 2) in response to SEA or heat-treated SEA (90°C, 10 min). All PS-exposing activity is lost in the heat-treated SEA. Values shown are mean $\pm$ SEM, $n = 9-15$. ** signifies $P < 0.01$, ANOVA.

consequence of the disease that has perhaps not been fully appreciated, in part because it is difficult to distinguish the effects of schistosome infection on red blood cells from those of concomitant heterologous infections. Schistosomiasis-associated anemia is almost certainly multifactorial, with several mechanisms likely responsible [4]. Factors proposed to play a role include anemia of inflammation [37], occult blood loss [38], and autoimmune hemolysis [39]. Based on studies relating increased levels of pro-inflammatory cytokines such as IL-6 to chronic schistosomiasis, anemia of inflammation is commonly cited as a major contributor to schistosomiasis-associated anemia. However, recent evidence suggests that it may not be a primary cause, and that other factors are at play [40]. In this report, we present evidence for another mechanism, SEA-induced erythrocyte cell death.

Erythrocyte cell death, or eryptosis [41], has as hallmarks several morphological and physiological changes, including alteration in Ca^{2+} homeostasis, changes in membrane permeability and integrity, and cell shrinkage. Erythrocyte cell death can be stimulated by various factors (see [13] for review), including osmotic shock, energy depletion, immunomodulators (anti-IgG, leukotriene, cyclosporine), metals (gold, lithium, silver), messenger molecules (PGE_2 , ceramide, platelet activating factor), anti-protozoan agents (amphotericin B, curcumin), and bacterial virulence factors (listeriolysin, peptidoglycan). Eryptosis is also associated with various clinical conditions [42], and may be involved in the survival of erythrocytes parasitized by the malaria pathogen *Plasmodium falciparum* [20, 21].

In this report, we demonstrate that *S. mansoni* SEA triggers specific markers of the cell death program in mouse erythrocytes, thus adding to this list a factor with potential relevance to morbidity from a major tropical disease that affects hundreds of millions of people worldwide. Erythrocytes incubated with 50–200 $\mu\text{g}/\text{ml}$ of SEA show a rise in intracellular Ca^{2+} levels, activation of caspase activity, and Ca^{2+} -dependent exposure of PS. Most of these effects are dose-dependent, though the activation of caspases occurs only at the highest concentration (200 $\mu\text{g}/\text{ml}$) tested. This result may indicate a limited role for caspases in SEA-induced erythrocyte cell death. Further, at high SEA concentrations (200 $\mu\text{g}/\text{ml}$), hemolysis also becomes a factor. Circulating antigens of approximately 0.1–3 $\mu\text{g}/\text{ml}$ of SEA have been measured in serum and urine samples from schistosome-infected individuals [43, 44] and are directly correlated with egg counts. However, the local concentrations of SEA in the

tissues and capillaries around the deposited eggs are thought to be far higher than circulating levels and are likely more relevant to evoking host responses. Indeed, the majority of anti-SEA immunoreactivity in the host is found in these areas [45–47]. The concentrations of SEA we are using in these experiments are similar to those used by others to evaluate effects of schistosome egg antigens on host immune responses [48].

Interestingly, SEA did not induce apoptotic cell shrinkage, another marker for erythrocyte cell death [22, 49]. Typically, higher cytosolic Ca^{2+} disrupts K^+ and Cl^- homeostasis by facilitating KCl efflux, paralleled by loss of water, leading to cell shrinkage. However, SEA had no detectable effect on erythrocyte cell size, as measured by forward scatter. Shrinkage-independent erythrocyte death has been observed previously by others [33–35], and could suggest the presence of channel inhibitors or ion binding proteins in SEA. Proteomic analysis of the *S. mansoni* egg secretome has identified 188 proteins, 16 of which fit into a class of proteins associated with ion binding activity [50]. These putative ion-binding (or other) secretory proteins could potentially interfere with cell volume regulation or other physiological processes.

Although severe anemia has been reported in chronically infected mice [51] we could not detect any significant changes in PS exposure from freshly drawn blood between control and infected erythrocytes (data not shown). One possible explanation for this observation could be the rapid clearance of PS-exposing erythrocytes from circulation *in vivo* and the heterogeneity of the erythrocyte population observed. Thus, more extensive experiments will be required to evaluate the relevance of these results *in vivo*. It is not clear what advantage, if any, would accrue to schistosomes from induction of erythrocyte cell death. One possibility is that signals secreted by the eggs that induce apoptosis in other cells coincidentally trigger erythrocyte cell death. Schistosome infections generate apoptotic responses in a variety of immune cells, such as splenocytes and CD4^+ cells. For example, SEA induces cell death of CD4^+ T cells through the binding of Fas ligand [26] and egg excretory/secretory products alter the Fas-Fas ligand system, a major apoptotic pathway, in mouse liver [27]. Other worm stages also have effects on host cell survival. For example, skin-stage schistosomes secrete a 23 kDa protein that appears to be pro-apoptotic for host CD4^+ and CD8^+ T cells [52]. However, it is also possible that specific induction of erythrocyte cell death provides an as yet unknown advantage to the parasite.

Our results suggest that the component in SEA that triggers erythrocyte cell death is heat labile, indicating it is likely to be a protein. The 188 proteins found in the *S. mansoni* egg secretome contain a group of cell death-related proteins engaged in various biological processes such as cell signaling, immunomodulation, redox balance, and other physiological processes [50]. Detailed studies of these secreted death proteins may lead to a better understanding of the molecular signals that regulate SEA-induced erythrocyte cell death, and, by extension, one pathway likely to lead to schistosomiasis-associated anemia. Understanding the elements of this pathway could provide clues for disrupting it, and perhaps strategies

to prevent the development of schistosomiasis-associated anemia.

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