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Schistosoma mansoni: Membranes from Adult Worms Reversibly Perturb Shape, Volume, and Membrane Organization of Intact Human Red Blood Cells

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THATTE, H. S., KASSCHAU, M. R., FURLONG, S. T., BYAM-SMITH, M. P., WILLIAMS, D. F., AND GOLAN, D. E. 1993. *Schistosoma mansoni*: Membranes from adult worms reversibly perturb shape, volume, and membrane organization of intact human red blood cells. *Experimental Parasitology* 76, 13–22. Adult forms of *Schistosoma mansoni* ingest host (human) red blood cells (RBCs). To elucidate potential mechanisms by which contact with adult parasites perturbs RBC membranes, we studied the effects of the membrane fraction of isolated schistosomes on RBC shape, volume, potassium ion content, and phospholipid and transmembrane protein lateral mobility. *S. mansoni*-treated RBCs exhibited rapid but spontaneously reversible shape change from discocytes to spheroechinocytes, reversible decrease in cell volume, and rapid loss of intracellular potassium ions. Treated RBCs also showed rapid but spontaneously reversible immobilization of membrane phospholipids and of band 3, the major transmembrane protein. These data suggest that components of adult *S. mansoni* membranes can perturb host RBC volume and membrane organization. In the absence of RBC lysis, RBC metabolic and repair mechanisms can reverse these effects. © 1993 Academic Press, Inc.

INDEX DESCRIPTORS AND ABBREVIATIONS: Band 3; Fluorescence photobleaching recovery; Phospholipid; Trematode; Eosin maleimide (EMA); Fluorescein phosphatidylethanolamine (Fl-PE); Fluorescence photobleaching recovery (FPR); High-performance liquid chromatography (HPLC); Lysophosphatidylcholine (LPC); Phosphate-buffered saline (PBS); Phosphatidylcholine (PC); Red blood cell (RBC).

INTRODUCTION

Schistosoma mansoni is a digenetic trematode that has a complex life cycle requiring a mammal, frequently a human, as its final host. A gastropod serves as the intermediate host, from which free-living cercariae are shed. Cercariae penetrate the skin of the final host, lose their tails, and immediately develop into schistosomula, which migrate first to the lungs and then to the liver. Within 6 weeks, adult male and female pairs reside in the host mesenteric veins. Schistosomes begin to ingest host red blood cells (RBCs) late in the first week after transformation from cercariae to schistosomula (Basch 1981; Bogitsh 1978; Bogitsh and Carter 1977). The availability of hemoglobin as a nutrient source depends on the ability of the worms to lyse host RBCs.

Human RBCs have been observed to adhere to the parasite tegument. These RBCs do not fuse with the worm but deform or lyse to form ghosts that remain tightly attached to the parasite surface (Caulfield and Cianci 1985). Such ghosts are morphologically abnormal, exhibiting membrane folding and surface redistribution of membrane proteins and lipids (Caulfield and Cianci 1985; Golan *et al.* 1986). Fluorescence photobleaching recovery (FPR) mea-

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surements demonstrate that two integral membrane proteins, band 3 and glycophorin, and two lipid analogues, fluorescein phosphatidylethanolamine (Fl-PE) and carbocyanine dyes, are immobilized in the membranes of adherent ghosts. These changes in RBC membrane properties are thought to be induced by transfer of lysophosphatidylcholine (LPC) from schistosomula to adherent RBCs (Golan *et al.* 1986). Similar alterations in RBC membrane morphology and dynamics are seen in RBCs treated with lytic concentrations of egg LPC or its major component, monopalmitoyl phosphatidylcholine (Golan *et al.* 1988).

The pellet (membrane) fraction of homogenates of whole adult *S. mansoni* also produces rapid lysis of human RBCs. The activity of the adult schistosome hemolytic agent is dependent on both temperature and pH (Kasschau and Dresden 1986). In the present study we investigate the effects of sublytic concentrations of this pellet fraction on the shape, size, potassium ion content, and membrane dynamics of intact human RBCs. We observe that RBCs incubated with the pellet fraction exhibit potassium efflux and rapid but reversible shape change, volume loss, and immobilization of band 3 and Fl-PE in the plane of the membrane. Whereas previous investigations have shown that schistosomula cause changes in RBC morphology and membrane dynamics associated with RBC lysis (Golan *et al.* 1986), the present study indicates that such changes are also induced in intact RBCs under sublytic conditions by adult forms of *S. mansoni*.

MATERIALS AND METHODS

Materials

Triton X-100 and bovine serum albumin were from Sigma (St. Louis, MO). Eosin maleimide (EMA) was from Molecular Probes (Eugene, OR). Fl-PE and phospholipid standards were purchased from Avanti Polar Lipids (Alabaster, AL).

Schistosome Membranes

Adult *S. mansoni* were perfused from heavily infected Syrian hamsters using established procedures (Duvall and Dewitt 1967). Worms were washed several times in perfusion buffer (140 m*M* NaCl, 20 m*M* Na citrate, pH 7.2) prior to homogenization in 120 m*M* Na citrate, pH 5.1. The homogenate was centrifuged at 25,000*g* for 30 min. The membrane fraction was resuspended in 120 m*M* Na citrate, pH 5.1, and incubated overnight at 37°C to maximize activation of hemolytic activity (Kasschau *et al.* 1986). Membranes were aliquoted and stored at −20°C for 3–4 months prior to use in experiments; hemolytic activity of the membranes was unaffected by these storage conditions (Kasschau *et al.* 1986). Protein concentration was determined according to Bensadoun and Weinstein (1976), using bovine serum albumin as the standard. Total phospholipid was determined according to Stewart (1980), using egg phosphatidylcholine (PC) as the standard. Membranes had a protein to phospholipid weight ratio of 1.5 ± 0.4 (means ± SEM).

The phospholipid composition of adult *S. mansoni* membranes was determined by high-performance liquid chromatography (HPLC), as described (Furlong and Caulfield 1988). In brief, lipids were extracted from parasite membranes using chloroform/methanol (Folch *et al.* 1957). Phospholipids were separated by HPLC using a 25 cm by 4.0 mm steel column packed with 5 μm Econosphere silica (Alltech Applied Sciences, State College, PA) on a Waters automated gradient HPLC system. The mobile phase consisted of acetonitrile/methanol/85% phosphoric acid (130:6:1.5) flowing at 1.25 ml/min. Phospholipids were detected at 214 nm by a Waters model 441 uv detector and identified by comparing retention times of sample peaks to those of unlabeled standards. Data were collected using a Nelson Model 760 or Model 960 interface and integrated using Nelson analytical software on a Hewlett–Packard (Palo Alto, CA) Vectra computer. Phospholipids present in worm membranes included PC, phosphatidylethanolamine, phosphatidylserine, and LPC (Fig. 1). The phospholipid composition of activated membranes was similar to that previously reported for adult *S. mansoni* membranes, except that significantly more LPC was present in activated membranes (Furlong and Caulfield 1988).

RBC Morphology

After informed consent was obtained, blood was drawn from three healthy volunteers into heparinized tubes. Blood was centrifuged at 600*g* and the plasma and buffy coat were removed. RBCs were washed three times with 120 m*M* Na citrate, pH 7.5 (citrate buffer), and used for assays within 30 min. Packed RBCs were resuspended to a hematocrit of 1% in citrate buffer containing 10 m*M* glucose. Adult *S. man-*

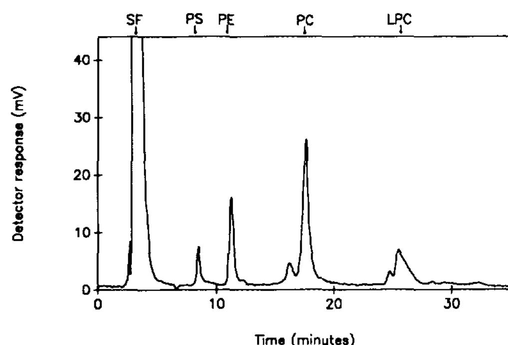


FIG. 1. HPLC chromatogram of phospholipids extracted from adult *Schistosoma mansoni* membrane fraction. Membranes were extracted with chloroform/methanol and separated using HPLC. Phospholipids were eluted isocratically from a silicic acid column with acetonitrile/methanol/85% phosphoric acid (130:6:1.5), detected at 214 nm using a uv detector, and identified by comparison to chromatograms of phospholipid standards. The major peak was phosphatidylcholine (PC); lesser quantities of phosphatidylserine (PS), phosphatidylethanolamine (PE), and lysophosphatidylcholine (LPC) were also found. SF, solvent front.

soni membrane fraction was added to the RBC suspension at a final concentration of 0.09 mg protein per milliliter. The mixture was vortexed and incubated at 37°C. Parallel samples of RBCs were treated with buffer alone. At specified times 20-μl aliquots of the suspension were removed, and RBCs were fixed in 1% glutaraldehyde in citrate buffer at room temperature for 30 min. Fixed RBCs were observed using a Zeiss Axioskop microscope with differential interference contrast optics and photographed at 160× magnification on Kodak TMax 400 black and white film. RBC diameter was measured from the photomicrographs. For each sample RBCs of different morphology (discocytes, spheroechinocytes) were measured in proportion to their relative abundance in the sample population, i.e., subpopulations with particular morphologies were not preferentially selected for RBC diameter measurements. Ten RBCs were measured at each time point in a single experiment; results from three similar experiments did not differ significantly from one another. Although fixed RBCs were routinely measured to ensure uniformity of sample handling and photomicrography, RBCs prior to fixation manifested identical morphologies and diameters to those in the same sample following fixation.

Measurement of RBC Potassium Ion Content

Packed RBCs were resuspended to a hematocrit of 0.7% in citrate buffer and the adult *S. mansoni* membrane fraction was added to the RBC suspension at a final concentration of 0.09 mg protein per milliliter. Control RBCs were incubated with buffer alone. The mixture was vortexed and incubated at 37°C. At 10-min intervals aliquots of the mixture were removed, centrifuged, resuspended in 120 m*M* LiCl, and centrifuged again through an *N*-butylphthalate layer to remove external Na^+ (Gunn and Tosteson 1971). The supernatant was discarded and the RBC pellet was lysed in 1% Triton X-100 and centrifuged at 14,000*g* for 1 min. RBC K^+ content was measured using flame photometry (Instrumentation Laboratories IL-443).

RBC Surface Labeling with Fluorescent Reagents

Membrane phospholipids. A solution of Fl-PE, 1 mg per milliliter in chloroform, was dried by evaporation, brought to a final concentration of 3 μg per ml in PBS, vortex mixed, and bath sonicated for 20 min. Washed RBCs were incubated with the Fl-PE solution for 30 min at room temperature. RBCs were then washed twice with citrate buffer containing 1% bovine serum albumin to remove excess label and were resuspended at 1% hematocrit in citrate buffer containing 10 m*M* glucose. Using this protocol Fl-PE was incorporated directly into the intact RBC membranes. The stoichiometry of labeling was 8×10^5 fluorophores per RBC, or 0.003 mole of probe per mole of endogenous membrane lipid (Golan *et al.* 1986).

Band 3. Band 3 was specifically labeled as described (Golan *et al.* 1986). Briefly, RBCs were incubated with EMA for 15 min at 21°C. Cells were then washed three times with PBS containing 1% bovine serum albumin and twice with PBS, and resuspended at 1% hematocrit in PBS containing 10 m*M* glucose. PBS was used instead of citrate buffer, because EMA-labeled RBCs tended to lyse in citrate buffer.

Fluorescently labeled RBCs were incubated at 37°C with the adult *S. mansoni* membrane fraction, 0.09 mg protein per milliliter (final concentration). Nine microliters of the mixture was immediately transferred to a glass slide, and a coverslip was placed on the suspension and sealed on the slide using vacuum grease.

Fluorescence Photobleaching Recovery (FPR)

The lateral mobilities of Fl-PE and of EMA-labeled band 3 were measured by FPR (Axelrod *et al.* 1976). Briefly, the plasma membrane of a single RBC was observed in a fluorescence microscope using a focused laser beam as the excitation source. A small area of membrane was exposed to a brief, intense laser pulse, causing irreversible bleaching of the fluorophore in that area. Fluorescence recovery, resulting from the lateral diffusion of unbleached fluorophore into the bleached area, was measured. Analysis of the fluores-

cence recovery curves yielded the fraction (f) of Fl-PE or band 3 molecules that were free to diffuse in the plane of the membrane and the diffusion coefficient (D) of the mobile fraction.

Our FPR apparatus and analytical methods have been described in detail (Golan *et al.* 1986; Caulfield *et al.* 1991). The Gaussian beam radius at the sample plane was 0.8 μm, as determined by using a two-dimensional emission scan technique (Stolpen *et al.* 1988). Photobleaching power at the sample was approximately 2 mW. Sample temperature was controlled to 37.0 ± 0.1°C by using a thermal stage. FPR experiments on intact RBCs were initiated within 2 min of incubation with the *S. mansoni* membrane fraction.

RESULTS

Adult S. mansoni *membranes induce alterations in RBC shape and volume.* Incubation at 37°C with the adult *S. mansoni* membrane fraction caused time-dependent changes in RBC morphology (Fig. 2). Within 5 min RBCs were transformed from discocytes to spheroechinocytes and the cells showed a marked decrease in RBC diameter (Fig. 3). After 20 min of incubation shrunken spherocytes, cells with abnormally folded membranes, and a few lysed RBCs were seen (Fig. 2b), and RBC diameter decreased to a minimum value (Fig. 3). A portion of the decrease in RBC diameter could be ascribed to the change in cell shape, since the diameter of a sphere containing 90 fl (the mean cell volume of normal RBCs) is 5.6 μm. The minimum diameter of RBCs treated with the *S. mansoni* membrane fraction was 4.6 μm, however, indicating that RBC volume loss as well as shape change must have occurred. The discocyte to spheroechinocyte transformation was found to be partially reversible on further incubation of cells at 37°C. Within 90 min the majority of RBCs reverted to a dis-

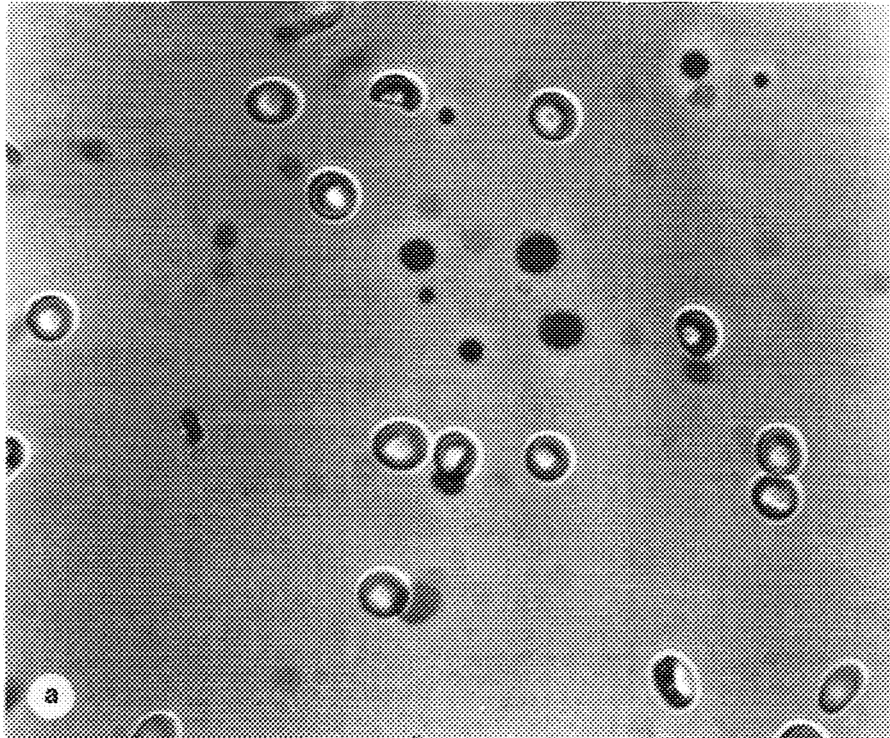
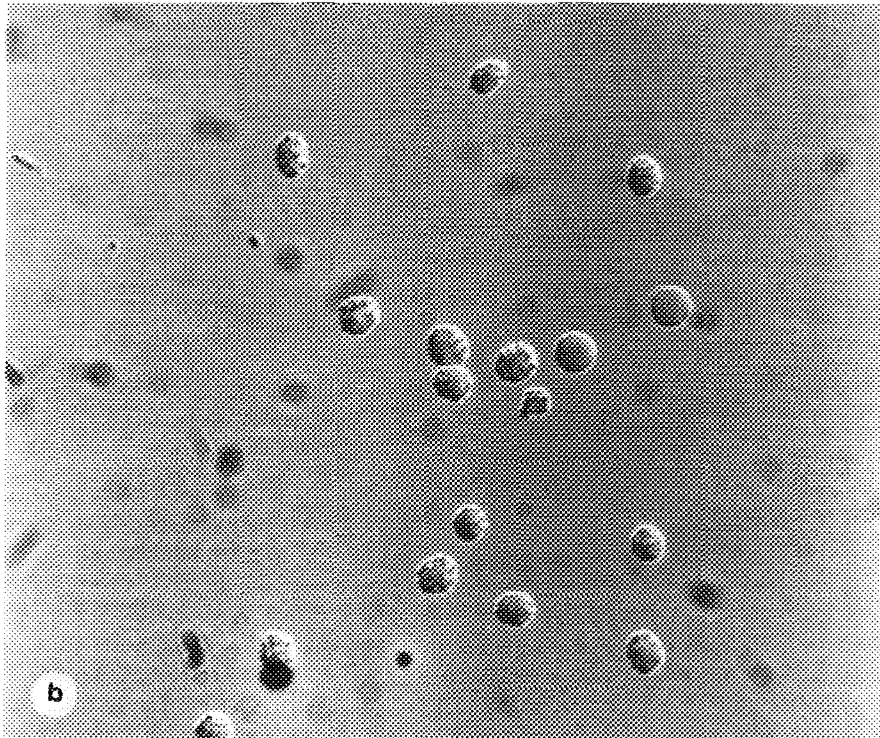


FIG. 2. Effect of adult *Schistosoma mansoni* membrane fraction on RBC shape. RBCs were incubated at 37°C with adult *S. mansoni* membrane fraction. At specified times cells were fixed in 1% glutaraldehyde and photographed. (a) Control RBCs. (b) RBCs treated for 20 min. (c) RBCs treated for 90 min. Magnification, 1000×.



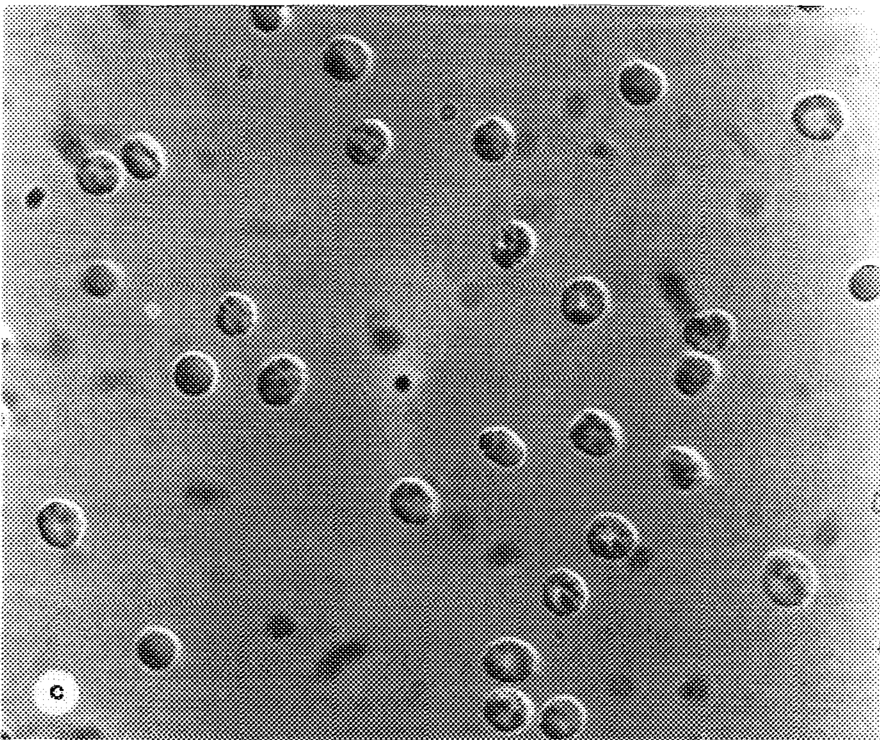


FIG. 2—*Continued*

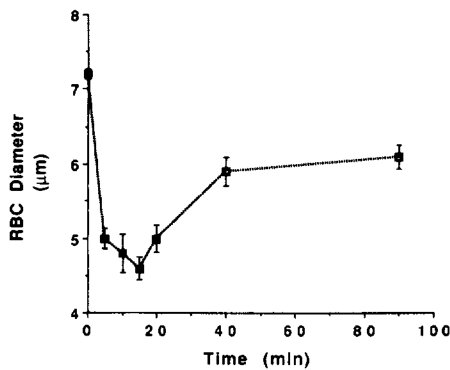


FIG. 3. Effect of adult *Schistosoma mansoni* membrane fraction on RBC diameter. RBCs were incubated at 37°C with adult *S. mansoni* membrane fraction. At specified times cells were fixed in 1% glutaraldehyde and photographed. RBC diameter was measured from the photomicrographs. Results are expressed as means ± SEM of 10 measurements at each time point.

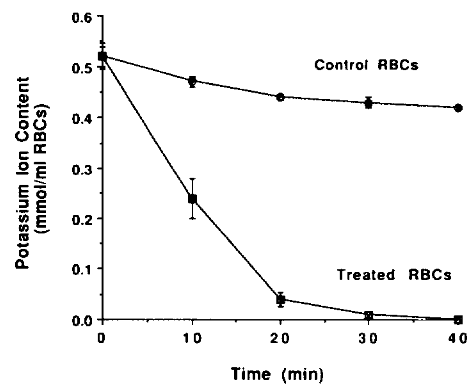


FIG. 4. Effect of adult *Schistosoma mansoni* membrane fraction on RBC potassium ion content. RBCs were incubated at 37°C with adult *S. mansoni* membrane fraction. Control cells were incubated with buffer alone. At 10-min intervals, samples were removed and RBCs were pelleted and dissolved in Triton X-100. RBC potassium ion content was measured by flame photometry. Data points represent means ± SEM of four independent measurements.

cocytic shape (Fig. 2c) and the cell diameter increased from 64 to 85% of control values (Fig. 3). Prolonged incubation of RBCs at 37°C in glucose-containing buffer did not result in complete reversibility of RBC shape and diameter changes, however (data not shown).

Adult S. mansoni *membranes induce loss of intracellular potassium ions from RBCs.* Incubation of RBCs at 37°C with the adult *S. mansoni* membrane fraction also caused rapid loss of intracellular potassium ions (Fig. 4). Sublytic concentrations of membrane fraction induced complete release of intracellular potassium ions within 30 min of incubation at 37°C. In contrast, the potassium ion content of control RBCs remained at greater than 80% of initial values over the duration of the experiment (Fig. 4). The kinetics of intracellular potassium loss were dependent on the concentration of the membrane fraction and the temperature of incubation. The rate of potassium loss at 25°C was fivefold slower than that at 37°C (data not shown).

Adult S. mansoni *membranes cause reversible immobilization of RBC membrane phospholipids and proteins.* The lateral mobility of a phospholipid analogue, Fl-PE, and the major transmembrane protein, band 3, were measured by FPR at 37°C in RBCs treated with sublytic concentrations of the adult *S. mansoni* membrane fraction. There was a time-dependent decrease in the fractional mobility of Fl-PE in treated RBCs (Fig. 5). Fl-PE mobility decreased from 88 to 74% within 1 min of incubation with *S. mansoni* membranes and reached a minimum value of 35–40% by 10 min of incubation. Prolonged incubation of treated RBCs at 37°C caused the fractional mobility of Fl-PE to revert to normal levels (Fig. 5). The kinetics of these changes were consistent with those of the *S. mansoni*-induced alterations in RBC shape and size (Figs. 2 and 3). Although treatment with the adult *S. mansoni* membrane fraction caused marked changes in Fl-PE fractional mobility, the diffusion coefficient of the phospholipid analogue remained constant over the experimental period. D values were $2.1 \pm 0.1 \times 10^{-9}$ cm^2 sec^{-1} (means ± SEM, n = 24) in treated RBCs and $2.0 \pm 0.1 \times 10^{-9}$ cm^2 sec^{-1} (means ± SEM, n = 24) in control RBCs.

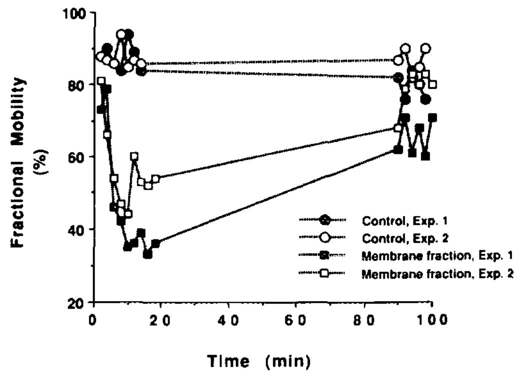


FIG. 5. Effect of adult *Schistosoma mansoni* membrane fraction on fractional mobility of RBC membrane phospholipids. Fl-PE-labeled RBCs were incubated at 37°C with buffer (control) or with adult *S. mansoni* membrane fraction. FPR was used to measure Fl-PE lateral mobility. Data points represent the pairwise average of two independent measurements. Abscissa, time of incubation (minutes). Ordinate, fractional mobility (%).

The adult *S. mansoni* membrane fraction rapidly immobilized band 3 in intact RBCs. This effect was reversible upon prolonged (120 min) incubation at 37°C (Fig. 6). The diffusion coefficient of band 3 was $2.5 \pm 0.2 \times 10^{-11}$ cm^2 sec^{-1} (means ± SEM, $n = 21$) in control RBCs and $1.6 \pm 0.2 \times 10^{-11}$ cm^2

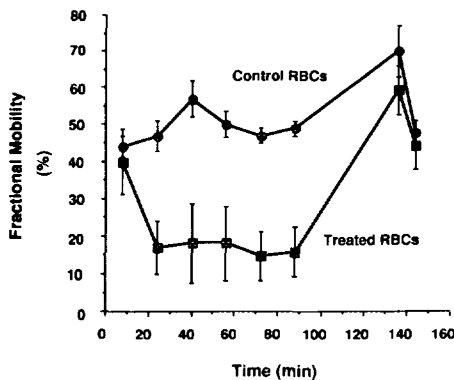


FIG. 6. Effect of adult *Schistosoma mansoni* membrane fraction on fractional mobility of band 3 in intact RBC membranes. Eosin maleimide-labeled RBCs were incubated at 37°C with buffer (control) or with adult *S. mansoni* membrane fraction. FPR was used to measure band 3 lateral mobility. Data points represent means ± SEM of four independent measurements. Abscissa, time of incubation (minutes). Ordinate, fractional mobility (%).

sec^{-1} (means ± SEM, $n = 6$) in RBCs treated for 120 min with *S. mansoni* membrane fraction. D values could not be measured reliably in RBCs treated for less than 90 min, since f values were less than 20% in these samples. The effect of adult *S. mansoni* membranes on the fractional mobility of band 3 was similar to that on the mobility of Fl-PE (Fig. 5). Since both lipid and protein mobilities were affected, it is likely that the adult *S. mansoni* membrane fraction induced a profound but reversible reorganization of RBC membrane structure.

DISCUSSION

In this report we find that, at sublytic concentrations, the membrane fraction of adult *S. mansoni* worm homogenate causes reversible changes in RBC shape, RBC volume, and the lateral mobility of band 3 and a fluorescent phospholipid analogue in the RBC membrane. All of these parameters are regulated by the macromolecular organization of the RBC membrane, suggesting that components of *S. mansoni* membranes perturb RBC membrane structure as well as function. Further, since the RBC changes are reversible with time, intact human RBCs appear to have the metabolic machinery necessary to repair the potentially toxic damage induced by *S. mansoni*.

We have previously shown that the membrane fraction of homogenates of whole adult *S. mansoni* produces rapid lysis of human RBCs at pH 5.1 (Kasschau and Dresden 1986). Adult *S. mansoni* membranes are found in the present study to induce profound morphological changes without lysis in intact RBCs at pH 7.5. The discocyte to spheroechinocyte shape transformation caused by adult *S. mansoni* membranes is also produced by ATP depletion, oxidative stress, and a wide variety of membrane reactive agents including LPC (Sheetz and Singer 1976). Since schistosomula of *S. mansoni* have been shown to synthesize and secrete LPC (Golan *et al.* 1986, 1988), it is likely that LPC is one com-

ponent of adult *S. mansoni* membranes responsible for RBC shape change. The partial reversibility of shape change with time in RBCs treated with *S. mansoni* membranes could be due to acylation of LPC to phosphatidylcholine by RBC enzymes, or to translocation of LPC from the outer to the inner leaflet of the RBC membrane. In separate experiments, incubation of RBCs with phosphatidylcholine did not produce the discocyte to spheroechinocyte shape transformation (data not shown).

Changes in RBC volume are often associated with alterations in membrane cation permeability leading to loss or gain of cell water (for a review see Hoffmann and Simonsen 1989). We find here that *S. mansoni* membranes induce rapid decreases in RBC volume and potassium ion content, suggesting that intracellular potassium loss could account for the change in cell volume. Increased membrane permeability to potassium ions could occur through the formation of nonspecific channels or pores in the membrane, or through activation of the KCl cotransport system (Hoffmann and Simonsen 1989) in treated cells. Activation of KCl cotransport has been demonstrated in osmotically swollen cells (Dunham and Ellory 1981), cells treated with *N*-ethylmaleimide (Lauf 1986), and cells from human subjects with sickle cell anemia or hemoglobin C disease (Brugnara *et al.* 1985, 1986). The mechanism by which KCl cotransport is activated in these cell types may involve changes in molecular interactions between integral and peripheral membrane components (for a review see Parker and Dunham 1989).

RBCs treated with adult *S. mansoni* membranes exhibit rapid but reversible decreases in the lateral mobilities of both band 3 and a phospholipid analogue. These effects parallel changes in RBC shape and volume induced by worm membranes and may be due to similar molecular mechanisms. The finding that both protein and lipid components are immobilized suggests that adult *S. mansoni* membranes cause a global reorganization of the RBC membrane. One mechanism that could underlie such a process is lateral phase separation of RBC membrane lipids, induced by transfer of lipids or proteins from the parasite to the RBC membrane. Other potential mechanisms that cause transmembrane protein immobilization, including protein aggregation or protein interactions with membrane skeletal components, are unlikely to affect the lateral mobility of membrane phospholipids.

The molecular components of adult *S. mansoni* membranes responsible for changes in RBC shape, volume, and membrane organization remain to be elucidated. Sublytic concentrations of LPC, a prominent phospholipid class in the activated membrane fraction, cause the RBC discocyte to spheroechinocyte shape transformation and RBC volume loss. LPC at sublytic concentrations has little effect on RBC potassium ion content or RBC membrane phospholipid lateral mobility, however (Golan *et al.* 1986; unpublished observations). Components of adult *S. mansoni* membrane other than (or in addition to) LPC appear to be required for alterations in potassium ion permeability and protein and phospholipid mobility in membranes of intact RBCs.

Schistosomula of *S. mansoni* induce RBC lysis accompanied by irreversible changes in RBC membrane morphology and protein and lipid dynamics (Golan *et al.* 1986). In contrast, RBC alterations in the present study are reversible. Intact RBCs appear to be capable of using metabolic and repair mechanisms to reverse membrane alterations induced by components of adult *S. mansoni* membranes. Such mechanisms could include lysophospholipid acylation, lipid translocation between outer and inner membrane leaflets, and proteolysis of parasite proteins incorporated into RBC mem-

branes. Finally, we note that our previous studies investigated the effects of schistosomula of *S. mansoni* on RBC membrane structure and function (Golan *et al.* 1986, 1988), whereas the present studies use adult forms of these parasites. Different stages of the *S. mansoni* life cycle may exhibit differential effects on host RBCs.

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