Schistosomula of *Schistosoma mansoni* Use Lysophosphatidylcholine to Lyse Adherent Human Red Blood Cells and Immobilize Red Cell Membrane Components

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**Abstract.** Human red blood cells (RBCs) adhere to and are lysed by schistosomula of *Schistosoma mansoni*. We have investigated the mechanism of RBC lysis by comparing the dynamic properties of transmembrane protein and lipid probes in adherent ghost membranes with those in control RBCs and in RBCs treated with various membrane perturbants. Fluorescence photobleaching recovery was used to measure the lateral mobility of two integral membrane proteins, glycophorin and band 3, and two lipid analogues, fluorescein phosphatidylethanolamine (FI-PE) and carbocyanine dyes, in RBCs and ghosts adherent to schistosomula. Adherent ghosts manifested 95-100% immobilization of both membrane proteins and 45-55% immobilization of both lipid probes. In separate experiments, diamide-induced cross-linking of RBC cytoskeletal proteins slowed transmembrane protein diffusion by 30-40%, without affecting either transmembrane protein fractional mobility or lipid probe lateral mobility. Wheat germ agglutinin- and polylysine-induced cross-linking of glycophorin at the extracellular surface caused 80-95% immobilization of the transmembrane proteins, without affecting the fractional mobility of the lipid probe. Egg lysophosphatidylcholine (lysoPC) induced both lysis of RBCs and a concentration-dependent decrease in the lateral mobility of glycophorin, band 3, and FI-PE in ghost membranes. At a concentration of 8.4 μg/ml, lysoPC caused a pattern of protein and lipid immobilization in RBC ghosts identical to that in ghosts adherent to schistosomula. Schistosomula incubated with labeled palmitate released lysoPC into the culture medium at a rate of 1.5 fmol/h per 10⁶ organisms. These data suggest that lysoPC is transferred from schistosomula to adherent RBCs, causing their lysis.

*Schistosoma mansoni* lives in intimate contact with human blood cells from the third or fourth day after infection. The surface of this parasite is unusual in that it is bound by a syncytium covered by two membranes (8, 31, 47). The outer membrane appears to be a lipid bilayer almost devoid of protein, since the membrane lacks intramembrane particles by freeze-fracture, whereas the inner membrane resembles the plasma membrane of mammalian cells (8, 31, 47). These membranes do not endocytose multivalent ligands but instead slough the ligands into the culture medium in vitro (42). Labeled surface glycolipids and glycoproteins are also released into the medium, suggesting that the outer membrane, at least, is continually being shed (41).

The tegumental membranes exhibit several unique phenomena in interactions with mammalian blood cells. Human neutrophils fuse with the parasite to form a hybrid membrane that is derived from the cell plasma membrane and the worm outer tegumental membrane (8). This hybrid membrane mixes with the normal outer membrane upon lysis of the neutrophil (9). Similar membrane fusions may be responsible for the parasite's acquisition of host membrane components such as murine histocompatibility antigens (23, 24, 43, 44) and membrane glycolipids (14, 15, 29). Further, human neutrophils endocytose multivalent ligands from the parasite surface along with the surface components to which the ligands are bound (10). Paradoxically, the removal of these surface components does not produce structural alteration in the parasite membranes as seen by freeze-fracture or thin-section transmission microscopy. Unlike neutrophils, human red blood cells (RBCs) adhering to the parasite do not fuse with the worm but lyse to form ghosts that remain attached to the parasite surface (6).

1. Abbreviations used in this paper: Con A, concanavalin A; diI, carbocyanine dyes; EMA, eosin-5-maleimide; FI-PE, fluorescein phosphatidylethanolamine; FPR, fluorescence photobleaching recovery; FTSC, fluorescein-5-thiosemicarbazide; lysoPC, lysophosphatidylcholine; PMSF, phenylmethyisulfonyl fluoride; RBC, red blood cell; WGA, wheat germ agglutinin.
The present studies investigate the mechanism of RBC lysis. Because cultures of schistosomula and RBCs contain a few adherent ghosts, many adherent and nonadherent RBCs, and an excess of parasite membrane, the membrane properties of individual cells and ghosts must be measured in situ. Fluorescence photobleaching recovery (FPR) is ideal for this purpose, because it measures the dynamic properties of protein and lipid in a single biological membrane without perturbation of the membrane environment (3). In this technique, a single cell membrane is observed in a fluorescence microscope, using a focused laser beam as the excitation source. A small area of the membrane is exposed to a brief, intense laser pulse, causing irreversible bleaching of the fluorophore in that area. Fluorescence recovery resulting from lateral diffusion of unbleached fluorophore into the bleached area is measured. Analysis of the fluorescence recovery curves yields the fraction of fluorescently labeled protein or lipid that is free to diffuse in the plane of the membrane (the mobile fraction, f), as well as the diffusion coefficient of the mobile fraction (D).

FPR is used to measure the lateral mobility of two integral membrane proteins, glycophorin and band 3, and two lipid probes, fluorescein phosphatidylethanolamine (FI-PE) and carboxyamine dyes (diI), in RBCs and ghosts adherent to schistosomes. These findings are compared with measurements obtained in RBCs subjected to a variety of membrane perturbants. Such experiments suggest that lysophosphatidylcholine (lysoPC) is the agent responsible for RBC lysis on the parasite surface. In independent experiments, worms are labeled with 3H-palmitate to see whether they metabolize palmitate to lysoPC and, if so, whether the lysoPC is released into the culture medium, as predicted from the shedding of the outer surface membrane.

Materials and Methods

Reagents

Fluorescein-5-thiosemicarbazide (FTSC), eosin-5-maleimide (EMA), and carboxyamine dyes (di-I-C3, di-C6, di-C12) were purchased from Molecular Probes, Inc. (Junction City, OR). Diamide and poly-L-lysine were from Sigma Chemical Co. (St. Louis, MO). Wheat germ agglutinin (WGA) and concanavalin A (Con A) were from Vector Laboratories (Burlinegam, CA). 9,10-Phthalic acid (specific activity, 27.5 Ci/mmol) was from New England Nuclear (Boston, MA). FI-PE and phospholipids (egg phosphatidylinositol, and bovine brain phosphatidylserine, all >99% pure) were from Avanti Polar-Lipids (Birmingham, AL). The fatty acid composition of egg lysoPC is 50.5% palmitic acid, 25.7% stearic acid, 18% oleic acid, and 0% palmitoleic acid (Avanti Polar-Lipids, Inc., personal communication). RPMI-1640 culture medium was from Gibco (Grand Island, NY). Sodium m-periodate and bovine serum albumin (essentially fatty acid and globulin free) (BSA) were from Sigma Chemical Co. EDTA was from Fisher Scientific Co. (Pittsburgh, PA). Phenylmethylsulfonyl fluoride (PMSP) was from Eastman Organic Chemicals (Rochester, NY).

Fluorescent Labeling of Human RBCs and RBC Ghosts

Glycophorin. RBC glycophorin was fluorescently labeled by conjugation of FTSC to glycophorin-linked (22) sialic acid moieties (27). Similar methods have been used by Abraham and Low (1) and by Cherry et al. (23) to label glycophorin selectively with other fluorescent probes. Fresh human blood was washed three times by centrifugation in phosphate-buffered saline (PBS) (128 mM sodium chloride, 10 mM sodium phosphate, pH 7.4) with 120 μM PMSP and 1 mM EDTA (PBS/PMSP/EDTA). Washed RBCs were stirred with an equal volume of 2 mM sodium periodate in PBS for 15 min at 0°C to oxidize selectively sialic acid exposed at the outer membrane surface (22). Unreacted periodate was removed by washing twice in 0.1 M glyceral in PBS (1) and once in PBS. The oxidized RBCs were incubated with an equal volume of FTSC, 0.5 mg/ml in PBS, for 60 min at 0°C, then washed three times in PBS with 1% BSA (PBS/BSA) (1) and once in RPMI. Unless otherwise indicated, all operations were performed at 4°C in the dark. Under these labeling conditions 80% of the membrane-associated fluorescence co-migrated on SDS polyacrylamide gels with the sialoglycoproteins PAS-1,2,3,4. Of this, 75% was coincident with glycophorin A (PAS-1,2) (21). The stoichiometry of labeling as determined by spectrophotometry and RBC membrane protein assay was 1.9 fluorophores per glycophorin monomer, or 1 x 10^10 fluorophores per RBC.

Band 3. RBC band 3 was fluorescently labeled by the technique of Nigg and Cherry (39). Briefly, fresh human RBCs washed in PBS/PMSP/EDTA were mixed with 0.2 mg/ml (final concentration) EMA in PBS with 10 mM glucose for 45 min at room temperature. Excess fluorophore was removed by washing three times in PBS/BSA and once in RPMI, all at 4°C. Under these conditions >80% of the membrane-associated fluorescence co-migrated on SDS polyacrylamide gels with band 3, as confirmed by selective extraction procedures on EMA-labeled RBC ghosts (39). The stoichiometry of labeling determined as above was 0.8 fluorophores per band 3 monomer, or 1 x 10^10 fluorophores per RBC.

Fi-PE. A modification of the technique of Golan et al. (25) was used to incorporate Fi-PE into the membranes of intact RBCs. Fi-PE (2 mg) was dried from a stock solution in chloroform, 10 μl of PBS was added, and the mixture was bath sonicated at room temperature until clarified. 75 μl of the Fi-PE mixture was added to 1.5 ml of washed, packed RBCs in 3.0 ml PBS and the mixture gently shaken at room temperature for 30 min. Labelled cells were washed three times in PBS/BSA and once in RPMI. The stoichiometry of labeling, determined by comparing the average fluorescence intensities of FiTSF- and Fi-PE-labeled RBCs, was 8 x 10^2 fluorophores per RBC or 0.003 μl of Fi-PE per mole of endogenous membrane lipid. This concentration of Fi-PE does not perturb the dynamic behavior of RBC membrane lipid (unpublished observations). In some cases Fi-PE-labeled RBCs were lysed in 40 μl of 5 mM sodium phosphate with 1 mM EDTA and 120 μM PMSP, pH 7.4, for 45 min at 4°C. The resulting ghosts were pelleted by centrifugation and washed three to four times in the lysis buffer and once in RPMI, all at 4°C.

diI. 50 μl of diI, 0.5 mg/ml in ethanol, was added to 0.5 ml of washed, packed human RBCs in 8.0 ml of PBS and the mixture gently shaken at 4°C for 30 min. Excess fluorophore was removed by washing twice in PBS to which 1% BSA had been added (PBS/1% BSA) and once in RPMI/1% BSA. Fi-PE-, FTSC-, and EMA-labeled RBCs each comprised a uniform population of fluorescent, intact discocytes, whereas diI labeled only 10% of the total RBCs and lysed ~50% of the RBCs.

Preparation of Schistosomula and Fluorescent Labeling with Carboxyamine Dyes

Schistosomula were prepared as described (40, 42). 2,000 schistosomula were cultured overnight at 37°C and mixed in 1.5 ml PBS with 2 μl of diI, 1.0 mg/ml in ethanol, for 30 min at room temperature. The labeled somules were washed twice in PBS and once in RPMI/0.1% BSA.

Incubation of Schistosomula with RBCs

1,000 cultured schistosomula were mixed with 2 μl of fluorescently labeled RBCs in 100-500 μl of RPMI/0.1% BSA and incubated at 37°C for 24-48 h. Controls were labeled RBCs or RBC ghosts incubated in the absence of somules. Immediately before FPR or photography, samples were treated with 10 mM eserine sulfate to immobilize the worms (42). This concentration of eserine sulfate did not affect the lateral mobility of RBC membrane components in control RBCs or ghosts.

Diamide Treatment of RBCs

RBCs were incubated with either 2 or 20 mM diamide in PBS at 37°C for 30 min (30, 45), washed twice in PBS containing 10% glyceral, and washed once in PBS. Diamide treatment was performed either before or after fluorescent labeling. Both protocols yielded the same FPR results.

Incubation of RBCs on Poly-l-lysine-, WGA-, and Con A-Coated Slides

FTSC-, EMA-, and Fi-PE-labeled RBCs were washed twice more in RPMI...
and resuspended in 20 vol of this buffer. Poly-U-lysine was prepared as a 1.0 mg/ml solution in H2O. WGA and Con A were prepared as 0.5 mg/ml solutions in PBS. 100–200 μl of these solutions were dropped on clean glass slides for 10–15 min at room temperature and the slides washed with PBS. 5 μl of fluorescently labeled RBC suspension was then dropped on the slides for 10 min at room temperature. The slides were washed with RPMI and examined immediately.

**Incubation of RBCs or Ghosts with LysoPC**

25 μl of packed, fluorescently labeled RBCs or RBC ghosts were mixed with 12 μl of PBS and 0–20 μg/ml of egg lysoPC from a freshly prepared stock solution of 10 mg/ml in H2O. The mixture was vortexed for 1 min at room temperature, washed twice in PBS and once in RPMI with 1% BSA (RPMI/BSA). Samples were diluted in 10 vol of RPMI/BSA and 1-μl aliquots were sealed on RPMI/BSA-treated microscope slides for FPR experiments. Pretreatment with BSA prevented echinocyte formation in control RBC samples.

**FPR**

A 4-W argon ion laser (164-08; Spectra-Physics Inc., Mountain View, CA) tuned to 488 nm was used as the excitation source for a fluorescence microscope (Leitz Orthoplan) equipped for incident-light (Ploem) illumination. The beam was focused to a waist at the secondary image plane of the microscope and by a weak planoconvex lens (f = 250, 350, or 500 mm) and to another waist at the specimen plane by a 100 or 63× phase fluorescence objective (Leitz). An interferometer (Ealing Corp., S. Natick, MA) placed in the excitation path was used to split the beam into measuring and bleaching paths, as described (25). The interferometer mirrors were fitted with remote-control linear actuators (Newport Corp., Fountain Valley, CA) to allow precise alignment of bleaching and measuring beams at the sample plane. The Gaussian beam radius at the sample plane, as determined by a two-dimensional emission scan technique (Brown, C. S., A. H. Stolpen, and D. E. Golan, manuscript submitted for publication), was 0.53 ± 0.02 μm. Photobleaching power at the sample was ∼2 mW. Bleaching times were typically 40 ms for protein diffusion measurements and 5 ms for lipid diffusion measurements. Measuring beam intensities were ∼3 μW. The optical apparatus was mounted on a 4′×6′ research quality vibration isolation table (Newport Corp.). Sample temperatures were controlled to ±0.1°C by using a thermal microscope stage (Leitz).

Emitted light was collected by the objective, filtered by the dichroic mirror (Leitz TK510) and suppression filter (Leitz K510), and directed through a series of lenses, beam splitters, and mirrors (Leitz MPV-3) to an extended S-20 photomultiplier tube (9558QA; Thorn EMI Gencom Inc., Fairfield, NJ) in a thermoelectrically cooled housing (TE-104RF; Products for Research, Inc., Danvers, MA) driven by a stabilized high-voltage power supply (HVS-1; Princeton Applied Research [PAR], Princeton, NJ). An adjustable field diaphragm placed in the image plane was used to discriminate against out-of-plane fluorescence. The photocurrent was fed into an amplifier/discriminator (PAR 121A), which converted the signal into a series of single-photon pulses. These pulses were counted within specified time intervals (typically 50–200 ms) by a photon counter (PAR 1099), and the number of counts per interval stored in a computer (PDP 11/23; Digital Equipment Corp., Marlboro, MA). The data could be converted into analog form for display on a cathode ray tube (4006-1; Tektronix, Inc., Beaverton, OR) and for hardcopy readout (Tektronix 4631), or plotted directly on an interactive digital plotter (Tektronix 4662/31). The computer was used to direct the sequence of opening and closing shutters for bleaching and measuring pulses, to gate the photon counter in step with the shutters, and to collect and analyze data. Data were fitted by nonlinear least squares analysis (4) to the approximate solution for fluorescence recovery after a “low percent” photobleach introduced by Yguerabide et al. (Eq. 12 of reference 50).

Unless otherwise indicated, samples were sealed on RPMI/1% BSA-treated microscope slides for FPR experiments. Blood was donated by four normal individuals. Experiments were performed two to five times and the results pooled, since there were no significant differences among results from different donors.

**Incubation of Schistosomula with Labeled Palmitate**

1 mCi of 9,10-3H-palmitic acid was dried under nitrogen, 200 μl of RPMI was added, and the mixture vortexed vigorously for 1 min. 60,000 schistosomula cultured overnight at 37°C were added to the radiolabel and the volume brought to 1.0 ml with RPMI/0.1% BSA. The incubation was carried out for 90 min at 37°C under a 5% CO2 atmosphere. After incubation the schistosomula were harvested by centrifugation at 200 g, washed three times in RPMI/0.1% BSA, and resuspended to 1,200 worms/ml in fresh RPMI/0.1% BSA. Aliquots consisting of 1.0 ml of the worm suspension per 1.5-ml conical centrifuge tube were incubated under a 5% CO2 atmosphere at 37°C for up to 12 h. Cultures were sampled at 0, 3, 6, and 9 h (two experiments) or 0, 6, and 12 h (one experiment). Either four (two experiments) or five (one experiment) tubes were pooled for each of three separate determinations at each time point. Somules were separated from the culture medium by centrifugation and both pellet and medium were analyzed for phospholipid.

**Phospholipid Analysis**

Medium and pellet were extracted in chloroform/methanol (17) and the phases separated by centrifugation. The upper phase was discarded and the lower phase immediately dried under nitrogen. Phospholipids from the lower phase were separated immediately using high performance liquid chromatography on a Waters automated gradient control system (Waters Instruments, Inc., Rochester, MN) equipped with a model 510 pump, a "Z" module, a U6K injector, a 10-μm Radial-Pak μPorasil cartridge, and a model 441 fixed wavelength detector. Samples were suspended in hexane/isopropanol/water (3/4/1), injected, and eluted isocratically with acetone/tetrahydrofuran/85% phosphoric acid (30/6/1.5) (22). Radioactivity was quantitated by liquid scintillation counting. Retention times for the various phospholipids were compared with those of standard phospholipids detected at a wavelength of 214 nm.

**Results**

**Adherence of Human RBCs to Schistosomula Leads to Lysis and Immobilization of Membrane Components**

Incubation with schistosomula for 24–48 h at 37°C caused many human RBCs to adhere to the worm surface and undergo profound morphological change (Fig. 1). Two general types of adherent RBCs were recognized. First, there were intact RBCs, many of which were deformed and attached by membrane tethers to the parasite surface. A few attached cells were echinocytic. Second, there were lysed RBCs (ghosts) which were visualized best by fluorescence microscopy (cf. Figs. 1 and 2 of reference 6). After a 24-h incubation three to five ghosts and 50–100 intact RBCs were attached to each organism. The ultrastructure of RBCs and ghosts adherent to schistosomula has been described (6).

The lateral mobilities of the major transmembrane proteins, band 3 and glycoporphin, and a phospholipid analogue, F1-PE, were measured at 23°C in both adherent RBCs and ghosts. The diffusion coefficients of both transmembrane proteins and F1-PE were two- to threefold less in deformed RBCs adherent to the parasite surface than in control RBCs incubated under the same conditions. The fractional mobilities of deformed and control RBCs were not significantly different (Table I). RBC ghosts on the worm surface exhibited 100% immobilization of both glycoporphin and band 3 and ~50% immobilization of F1-PE (Figs. 2 and 3; Table I). Since 100% of the membrane protein was immobilized, both adherent and non-adherent portions of the RBC ghost membranes must have been perturbed by the RBC–parasite interaction.

Hypotonically lysed RBC ghosts incubated in the absence of schistosomula for 24 h at 0°C manifested the slow protein diffusion and rapid lipid diffusion previously observed in intact RBCs and ghosts (5, 25, 28, 33, 34, 46). Unlike the ghosts found on the surface of schistosomula, these controls had fractional mobilities of ~60% for glycoporphin, 20% for
Figure 1. Fluorescence micrograph of a schistosomulum incubated with FTSC-labeled RBCs (R). Many of the adherent cells are deformed and attached to the parasite by tethers (arrows). Some cells are deformed into echinocytes (E) and others are lysed to form ghosts (G). Both echinocyte formation and lysis are observed after lysoPC treatment of intact RBCs. Bar, 10 μm.

Table I. Effect of Adherence to Schistosomula on Lateral Mobility of RBC Membrane Components  

<table>
<thead>
<tr>
<th>Glycophorin</th>
<th>Band 3</th>
<th>Fl-PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>f</td>
<td>n</td>
</tr>
<tr>
<td>Intact RBC control, 37°C</td>
<td>3.6 ± 1.7*</td>
<td>56 ± 12</td>
</tr>
<tr>
<td>Adherent deformed RBC</td>
<td>1.4 ± 0.5</td>
<td>71 ± 28</td>
</tr>
<tr>
<td>Adherent RBC ghost</td>
<td>-</td>
<td>2 ± 3</td>
</tr>
<tr>
<td>RBC ghost control, 37°C</td>
<td>47 ± 21</td>
<td>98 ± 6</td>
</tr>
<tr>
<td>RBC ghost control, 0°C</td>
<td>1.1 ± 0.4</td>
<td>63 ± 20</td>
</tr>
</tbody>
</table>

*Mean ± SD.

Glycophorin was labeled with FTSC, band 3 with EMA, and RBCs with Fl-PE. Schistosomula were mixed with fluorescently labeled RBCs and incubated for 24 h at 37°C. As controls, intact RBCs and hypotonically lysed RBC ghosts were incubated in the absence of worms for 24 h at either 37°C or 0°C. Lateral mobility at 23°C was measured by FPR. D, diffusion coefficient, ×10^11 cm^2 s^-1; f, fractional mobility, %. n, number of measurements. -, D cannot be determined for f < 20%.

Cross-linking Agents Do Not Immobilize an RBC Membrane Lipid Probe

Cross-linking agents were tested on RBCs for their ability to duplicate the pattern of protein and lipid immobilization observed in ghosts adherent to schistosomula. Diamide at a concentration of 2 mM has been shown to cross-link the cytoskeletal protein spectrin into a high molecular weight complex (30) and to cause a slowing of transmembrane protein diffusion in the RBC membrane (45). Both glycophorin and band 3 diffusion were slowed by 30-50% after treatment with 2 mM diamide (Table II), in agreement with earlier findings (45). Diamide had no effect, however, on the rate of Fl-PE diffusion or on the fractional mobility of either protein or lipid. Treatment with 20 mM diamide yielded identical FPR results (data not shown), suggesting that the effects of 2 mM diamide were maximal. Intracellular cross-linking of the RBC cytoskeleton is unlikely to cause the immobilization of transmembrane proteins and Fl-PE seen in ghosts adherent to schistosomula.

In an attempt to mimic the attachment of RBCs to the surface of schistosomula, RBCs were incubated on glass slides.
coated with cross-linking reagents. Both polylysine, which primarily binds to glycophorin-linked sialic acid residues, and WGA, which primarily binds to glycophorin-linked N-acetylglucosamine residues (2), should cross-link transmembrane glycophorin molecules on the external surface of the RBC membrane. Treatment with both of these agents lysed all RBCs adherent to the slide. As shown in Table II, polylysine and WGA partially immobilized glycophorin and band 3, each of which manifested a fractional mobility of ~5–20%. FI-PE fractional mobility was unaffected. Con A, which primarily binds to band 3–linked mannose residues (16, 20) and should cross-link transmembrane band 3 mole-

Table II. Effect of Cross-linking Agents on Lateral Mobility of RBC Membrane Components

<table>
<thead>
<tr>
<th>RBC Treatment</th>
<th>Glycophorin</th>
<th>Band 3</th>
<th>FI-PE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$D$ ($\times 10^{-11}$ cm$^2$ s$^{-1}$)</td>
<td>$f$ (%)</td>
<td>$n$</td>
</tr>
<tr>
<td>Intact control</td>
<td>2.3 ± 1.3*</td>
<td>82 ± 15</td>
<td>29</td>
</tr>
<tr>
<td>Hypotonic lysis</td>
<td>1.8 ± 0.6</td>
<td>62 ± 15</td>
<td>8</td>
</tr>
<tr>
<td>Diamide, 2 mM</td>
<td>1.8 ± 0.7</td>
<td>92 ± 5</td>
<td>12</td>
</tr>
<tr>
<td>Polylysine slide</td>
<td>---</td>
<td>6 ± 10</td>
<td>8</td>
</tr>
<tr>
<td>WGA slide</td>
<td>1.2 ± 7</td>
<td>21 ± 7</td>
<td>7</td>
</tr>
<tr>
<td>Con A slide</td>
<td>1.9 ± 0.4</td>
<td>65 ± 9</td>
<td>7</td>
</tr>
</tbody>
</table>

Glycophorin was labeled with FTSC, band 3 with EMA, and RBCs with FI-PE. Labeled RBCs were incubated under various conditions, as described in Materials and Methods. Lateral mobility was measured by FPR. All FPR measurements were performed at 23°C except those for glycophorin and band 3 mobility in the intact control and diamide-treated samples, which were performed at 37°C. $D$, diffusion coefficient, $\times 10^{11}$ cm$^2$ s$^{-1}$. $f$, fractional mobility, %. $n$, number of measurements. ND, not done. ---, $D$ cannot be determined for $f < 20\%$. *Mean ± SD.
Figure 3. Representative FPR curves of FI-PE lateral mobility in intact control RBCs (a) and in RBC ghosts adherent to schistosomula (b). FI-PE-labeled RBCs were incubated for 24 h at 37°C in the absence (a) and in the presence (b) of schistosomula. Experimental data are presented and analyzed as described in the legend to Fig. 2. Adherent RBC ghosts exhibited significant immobilization of FI-PE. (a) $D = 4.21 \times 10^{-9}$ cm$^2$ s$^{-1}$; $f = 96.1\%$ (b) $D = 2.03 \times 10^{-9}$ cm$^2$ s$^{-1}$; $f = 49.0\%$.

Three Carbocyanine Dyes Are Immobilized in Ghosts Adherent to Schistosomula

Carbocyanine dyes have been used in FPR experiments on both the schistosome membrane (32) and the RBC membrane (5, 33, 46), and they have been shown to transfer from adherent RBCs to the parasite membrane (6). RBCs were labeled with dils having acyl chain lengths of 12, 14, and 18 carbons, and then incubated with schistosomula. All three dyes manifested fractional mobilities of 80–90\% in deformed RBCs and 40–50\% in ghosts adherent to the parasite (Table III). The fractional mobility of dil in control RBCs or RBC ghosts incubated in the absence of worms was 90–100\% (Table III). The fractional mobility of dil in both adherent and control RBCs and RBC ghosts was similar to that of FI-PE (Table I). The diffusion coefficient of dil in RBC controls (Table III) was between two- and fivefold greater than that of FI-PE in control cells (Table I). This difference may have been caused either by the different incubation times at 37°C of the two preparations (48 h and 24 h, respectively) or by the dil(3) probes themselves, which may disrupt membrane architecture (5, 33). The lateral mobility of dil was also measured in schistosome membranes in which dye had been acquired either through incubation with labeled RBCs or through direct labeling. The fractional mobility of all three dil species was 65–80\%, and was independent of the method by which the parasite acquired the dye (Table III). Similarly, the diffusion coefficients of dil-C14 and dil-C18 were not significantly different in the two schistosome preparations (Table III).

LysoPC Induces Lysis of RBCs and Causes a Concentration-dependent Immobilization of RBC Membrane Components

Since intracellular and extracellular RBC membrane protein...
Table III. Effect of Adherence to Schistosomula on Lateral Mobility of Carboxyamine Dyes in RBCs

<table>
<thead>
<tr>
<th></th>
<th>dil-C12(3)</th>
<th>dil-C14(3)</th>
<th>dil-C18(3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D</td>
<td>f</td>
<td>n</td>
</tr>
<tr>
<td>Intact RBC control</td>
<td>20.1 ± 10.5*</td>
<td>95 ± 7</td>
<td>3</td>
</tr>
<tr>
<td>Adherent deformed RBC</td>
<td>ND</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td>Adherent RBC ghost</td>
<td>5.2 ± 3.6</td>
<td>48 ± 13</td>
<td>6</td>
</tr>
<tr>
<td>RBC ghost control</td>
<td>7.2 ± 1.1</td>
<td>102 ± 4</td>
<td>3</td>
</tr>
<tr>
<td>Schistosomula membrane (acq.)</td>
<td>12.9 ± 7.5</td>
<td>66 ± 1</td>
<td>3</td>
</tr>
<tr>
<td>Schistosomula membrane (dir.)</td>
<td>ND</td>
<td>ND</td>
<td>3</td>
</tr>
</tbody>
</table>

Schistosomula were mixed with diI-labeled RBCs and incubated for 48 h at 37°C. Lateral mobility at 23°C was measured by FPR. The lateral mobility of diI acquired by the worm membrane during the incubation with labeled RBCs is shown (acq.), as well as that of diI incorporated directly into the worm membrane (dir.). D, diffusion coefficient, ×10⁹ cm² s⁻¹; f, fractional mobility, %. n, number of measurements. ND, not done.

* Mean ± SD.

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Figure 4. High performance liquid chromatography chromatograms of ³H-palmitate-labeled lipid from intact schistosomula (a) and culture medium (b). Labeled lipid was eluted from a 10-μm silica cartridge (µPorasil) with acetonitrile/methanol/85% phosphoric acid (130/6/1.5) at a flow rate of 4.0 ml/min. Fractions were collected at 30-s intervals and radioactivity quantitated by liquid scintillation counting. Lipid standards were detected by ultraviolet absorption at 214 nm. Schistosomula released significant quantities of lysoPC into the incubation medium.

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Schistosomula Synthesize LysoPC and Release It Into the Culture Medium

Independent experiments were performed to determine whether schistosomula metabolize labeled palmitate to lysoPC. Fig. 4, a and b show typical chromatograms of ³H-phospholipids in cultured schistosomula and in the culture medium, respectively, after incubation for 90 min with ³H-palmitate and culture for 6 h in cold medium. In the worms one major phospholipid peak was observed and this peak co-eluted with a PC standard. In the culture medium there were two major phospholipid peaks and these co-eluted with PC and lysoPC standards. At this time point lysoPC comprised <2% of the total labeled phospholipid in intact schistosomula, while it was 34% of the total labeled phospholipid in the culture medium. The rate of appearance of labeled cross-linking techniques could not duplicate the immobilization of lipid probes in RBC ghosts adherent to schistosomula, we examined the effects of lysoPC on RBCs. This lyso- genic agent directly perturbs the RBC lipid bilayer and therefore might immobilize membrane lipid as well as transmembrane protein. Low concentrations of egg lysoPC (1.0–1.5 μg/ml) caused lysis of 50% of intact RBCs after 1-min incubation at room temperature. Higher concentrations of egg lysoPC (≥4 μg/ml) caused lysis of 100% of intact RBCs. In intact discocytes incubated in the absence of lysoPC, glycoporphin and band 3 had identical diffusion coefficients, D = 1–2 × 10⁻¹¹ cm² s⁻¹, and fractional mobilities (f) of 68 and 44%, respectively. Control values for FITC-PE mobility were D = 5 × 10⁻⁹ cm² s⁻¹ and f = 96% (Table IV). As the concentration of egg lysoPC was increased from 0 to 8.4 μg/ml, there was a monotonic decrease in the fractional mobility of glycoporphin, without change in the diffusion coefficient of the mobile fraction. Complete immobilization of both glycoporphin and band 3 (f < 10%) was observed at a lysoPC concentration of 8.4 μg/ml (Table IV). Both the diffusion coefficient and the fractional mobility of FITC-PE decreased monotonically over the same concentration range, although ~40% of the lipid probe remained mobile at a lysoPC concentration, 8.4 μg/ml, which had totally immobilized the transmembrane proteins. A higher concentration of egg lysoPC, 16.8 μg/ml, induced complete immobilization of the lipid probe as well (Table IV). These effects were not a direct consequence of lysoPC-induced RBC lysis, since identical results were obtained upon lysoPC incubation of RBC ghosts which had been produced by osmotic lysis (data not shown).
The changes observed in ghosts adherent to schistosomula are dependent on the immobilization of both transmembrane proteins of band 3 and glycophorin. Extracellular cross-linking results in the more extreme lateral mobility changes seen in adherent ghosts. Electron-dense plaques, but most of the ghost membrane is not directly attached to the parasite outer membrane (6). The finding that glycophorin and band 3 are completely immobilized in adherent ghosts suggests that the entire RBC membrane is affected, in both attached and unattached regions. Further, the slowed diffusion of transmembrane proteins and FI-PE in deformed, adherent RBCs implies that the critical interaction occurs at the parasite surface. Moreover, while the rate of release of lysoPC by schistosomula is too low to attain lytic concentrations in the medium, it is adequate to achieve a lytic amount of lysoPC at the worm surface. In a 24-h incubation each somule releases ~2 × 10^7 labeled lysoPC molecules per 10^3 organisms. In comparison, ~2 × 10^7 lysoPC molecules per RBC are required for lysis (49), and 1-10 × 10^6 lysoPC molecules per RBC are needed to produce echinocytes (18, 35, 38). Since the labeled palmitate experiments used a pulse rather than continuous labeling and are not corrected for pool size, it is likely that each somule produces enough lysoPC in a 24-h incubation to lyse three to five RBCs on its surface.

Labeled lysoPC is found in large amounts relative to PC in the culture medium, but in only trace quantities in total worm lipid. Labeled phosphatidylcholine in the culture medium may reflect the phospholipid composition of the outer (and, possibly, the inner) tegumental membrane, since surface labeled glycoproteins and glycolipids are shed by schistosomula into the medium (41). Parasite glands may also discharge lipid into the medium, although the effects of such lipids would presumably be manifested in non-adherent as well as adherent RBCs. The metabolic labeling is therefore consistent with the notion that lysoPC is present in the outer membrane, from which it can be transferred into a juxtaposed membrane or shed into the medium.

Lysed RBCs adhere to schistosomula via 10-20-nm thick electron-dense plaques, but most of the ghost membrane is not directly attached to the parasite outer membrane (6). The finding that glycophorin and band 3 are completely immobilized in adherent ghosts suggests that the entire RBC membrane is affected, in both attached and unattached regions. Further, the slowed diffusion of transmembrane proteins and FI-PE in deformed, adherent RBCs implies that the more extreme lateral mobility changes seen in adherent ghosts may reflect the phospholipid composition of the outer tegumental membrane. Labeled phosphatidylcholine, from which it can be transferred into a juxtaposed membrane or shed into the medium.

Discussion

The present studies demonstrate that RBC ghosts adherent to schistosomula exhibit complete immobilization of two integral membrane proteins, glycophorin and band 3. Two lipid probes, fluorscein-phosphatidyethanolamine and diI, are partially immobilized. These findings are not duplicated by membrane protein cross-linking techniques. An identical pattern of protein and FI-PE immobilization is found in RBCs lysed with egg lysoPC at a concentration of 8.4 µg/ml. Finally, parasites incubated with labeled palmitate release lysoPC into the culture medium at a rate of 1.5 fmol/h per 10^3 organisms. Taken together, these data suggest that lysoPC is transferred from schistosomula to adherent RBCs, causing their lysis.

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LysoPC is probably a component of the outer tegumental membrane of schistosomula which transfers directly into the plasma membrane of attached RBCs to produce lysis. Exchange of lysoPC between membranes of different cell types has been observed in other systems (48). When RBC adherence to schistosomula was promoted with lectins, lysis occurred within 1 h (6). Close apposition of parasite and RBC membranes is apparently critical for lysis. In the present studies both lysis of RBCs and immobilization of RBC membrane components are seen only in attached RBCs, again implying that the critical interaction occurs at the parasite surface. Furthermore, while the rate of release of lysoPC by schistosomula is too low to attain lytic concentrations in the medium, it is adequate to achieve a lytic amount of lysoPC at the worm surface. In a 24-h incubation each somule releases ~2 × 10^7 labeled lysoPC molecules into the medium. In comparison, ~2 × 10^7 lysoPC molecules per RBC are required for lysis (49), and 1-10 × 10^6 lysoPC molecules per RBC are needed to produce echinocytes (18, 35, 38). Since the labeled palmitate experiments used a pulse rather than continuous labeling and are not corrected for pool size, it is likely that each somule produces enough lysoPC in a 24-h incubation to lyse three to five RBCs on its surface.

Table IV. Effect of Egg LysoPC on Lateral Mobility of RBC Membrane Components

<table>
<thead>
<tr>
<th>Lysophosphatidylcholine µg/ml</th>
<th>Glycophorin</th>
<th>Band 3</th>
<th>FI-PE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D</td>
<td>f</td>
<td>n</td>
</tr>
<tr>
<td>0</td>
<td>1.2 ± 0.3*</td>
<td>68 ± 5</td>
<td>5</td>
</tr>
<tr>
<td>2.1</td>
<td>1.2 ± 0.7</td>
<td>35 ± 13</td>
<td>9</td>
</tr>
<tr>
<td>4.2</td>
<td>1.2 ± 0.6</td>
<td>22 ± 6</td>
<td>6</td>
</tr>
<tr>
<td>8.4</td>
<td>~</td>
<td>9 ± 7</td>
<td>9</td>
</tr>
<tr>
<td>16.8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Egg lysoPC was incubated with fluorescently labeled RBCs for 1 min at room temperature in RPMI, and lateral mobility at 23°C was measured by FPR. D, diffusion coefficient, × 10^11 cm^2 s^-1; f, fractional mobility, %; n, number of measurements. ND, not done. ~, D cannot be determined for f < 20%.

* Mean ± SD.


