The Lateral Diffusion of Lipid Probes in the Surface Membrane of *Schistosoma mansoni*

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Abstract. The technique of fluorescence recovery after photobleaching was used to measure the lateral diffusion of fluorescent lipid analogues in the surface membrane of *Schistosoma mansoni*. Our data reveal that although some lipids could diffuse freely others exhibited restricted lateral diffusion. Quenching of lipid fluorescence by a non-permeant quencher, trypan blue, showed that there was an asymmetric distribution of lipids across the double bilayer of mature parasites. Those lipids that diffused freely were found to reside mainly in the external monolayer of the outer membrane whereas lipids with restricted lateral diffusion were located mainly in one or more of the monolayers beneath the external monolayer. Formation of surface membrane blebs allowed us to measure the lateral diffusion of lipids in the membrane without the influence of underlying cytoskeletal structures. The restricted diffusion found on the normal surface membrane of mature parasites was found to be released in membrane blebs. Quenching of fluorescent lipids on blebs indicated that all probes were present almost entirely in the external monolayer. Juvenile worms exhibited lower lateral diffusion coefficients than mature parasites: in addition, the lipids partitioned into the external monolayer. The results are discussed in terms of membrane organization, cytoskeletal contacts, and biological significance.

*SCHISTOSOMA MANSONI* is one of the parasites responsible for the widespread chronic tropical disease bilharzia. The life cycle of this species of *Schistosoma* involves excretion of eggs liberated by adult worms in the primary (human) host, infection of aquatic snails (the secondary host), emergence from the snails of infective waterborne cercariae, which penetrate human skin and then develop into schistosomula, and finally adult worms. Infection is chronic, and the adults can reside for many years in the hepato-portal system of the host. The host–parasite combination exhibits the phenomenon of concomitant immunity, the essence of which is that chronically infecting adult worms elicit but resist an immune response that is nevertheless capable of partially protecting against subsequent infection (41, 42).

The molecular basis for concomitant immunity is not known, but it is generally thought that the evasion of immune damage is related to some property or properties of the schistosome surface. The outermost cell layer of schistosomes is a 2–4-μm thick syncytium known as the tegument. The innermost membrane of the tegument is a conventional bilayer (the basal membrane), whereas the outer membrane on the exterior of the syncytium consists of two tightly apposed bilayers that make a tetralayer (17, 18, 20). We shall refer to the two bilayers of the outer membrane as the inner and outer bilayers, each of which is composed of inner and outer monolayers.

A double outer membrane is an invariable and unique feature of intravascular trematodes (27). The double outer membrane is also present in schistosomula, having developed by about 3 h after skin penetration by cercariae (19). Since young schistosomula are susceptible to immune attack (39), the double outer membrane is not of itself sufficient to confer protection, although it may well be necessary. Other possible mechanisms for evasion of the immune response include the masking of surface antigens (39) and continuous sloughing of the outer membrane into the blood stream (32, 33). A further possibility, indicated by our earlier work (22) and explored more fully in this present study, is that the components of the outer membrane of adult schistosomes have restricted lateral diffusion in the plane of the membrane. Such restriction of lateral diffusion could inhibit immune attack in several ways (see Discussion).

The study reported here describes the measurement of the lateral diffusion coefficients of a variety of fluorescent lipid probes inserted into the outer membrane of *Schistosoma mansoni*, using the fluorescence photobleaching recovery (FPR) method (2, 23). We also explored the location of various probes in the bilayers of the outer membrane using a non-permeant fluorescence quencher, trypan blue. We have

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studied the outer membrane not only in intact worms but also after it has been lifted clear of underlying structures by formation of blebs (24). There is strong evidence, reviewed by Webb (48), that cytoskeletal interactions with membrane can impose severe constraints on the lateral diffusion of membrane proteins. It has been demonstrated that the formation of blebs or vesicles in certain eucaryotic systems such as skeletal muscle and lymphocytes releases these constraints thus enabling the lateral diffusion coefficient $D_L$ and the mobile fraction to approximate to the high values observed in simple model systems such as liposomes (44). We have observed a similar release of restricted lateral diffusion of lipid probes in surface blebs of $S. mansoni$. A possible mechanism for this is discussed.

Materials and Methods

Fluorescent Lipid Probes

The following lipid probes were used: 1,1'-di(octadecyl)-3,3,3',3' tetramethylindocarbocyanine perchlorate (C8-diII); 1,1'-di(hexacontanyl)-3,3,3',3' tetramethylindocarbocyanine perchlorate (C36-diII); 5-N-docosylamino-1,2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine perchlorate (C20-diI); N-fluorescein 1'-phosphatidylethanolamine (Fl-PE); N-fluorescein 1'-phosphatidylinositol (Fl-PI); N-fluorescein 1'-phosphatidylcholine (Fl-PC); N-fluorescein 1'-phosphatidylserine (Fl-PS). The Fl-PE band was scraped off the plates, eluted with CHCl₃-MeOH (2:1, vol/vol), and developed at room temperature with acetone/water (85:15, vol/vol). Visualization was by fluorescence under an ultraviolet strip-lamp. In the prepurification system the Rf values (i.e., distance migrated by the visualized spot relative to the solvent front) for fluorescein and Fl-PE were 1.0 and 0.45, respectively. The Fl-PE band was synthesized using a modification of the method of Fung and Stryer (15). Fluorescein isothiocyanate (60 mg) was added to a solution of 1,2-dipalmitoyl-sn-glycerol-3-phosphatidylethanolamine in 0.75 ml of CHCl₃ and 0.05 ml of triethylamine. The mixture was stirred gently for 2 h at room temperature, and washed three times with 20 ml of water to remove any unreacted fluorescein isothiocyanate or water-soluble products. The course of the reaction was followed by analyzing small samples by thin layer chromatography using silica gel plates developed with CHCl₃/MeOH/H₂O (65:25:4, vol/vol/vol). The washed reaction mixture was applied to several 20 × 20 cm silica gel thin layer chromatography plates (gel thickness = 0.25 mm) and developed at room temperature with acetone/water (65:15, vol/vol). Visualization was by fluorescence under an ultraviolet strip-lamp. In the preparative system the $R_f$ values (i.e., distance migrated by the visualized spot relative to the solvent front) for fluorescein and Fl-PE were 1.0 and 0.45, respectively. The Fl-PE band was scraped off the plates, eluted with CHCl₃/MeOH (2:1, vol/vol), taken to dryness under N₂ at room temperature, and washed three times with 20 ml of water to remove any unreacted fluorescein isothiocyanate or water-soluble products. The course of the reaction was followed by analyzing small samples by thin layer chromatography using silica gel plates developed with CHCl₃/MeOH/H₂O (65:25:4, vol/vol/vol). The washed reaction mixture was applied to several 20 × 20 cm silica gel thin layer chromatography plates (gel thickness = 0.25 mm) and developed at room temperature with acetone/water (65:15, vol/vol). Visualization was by fluorescence under an ultraviolet strip-lamp. In the preparative system the $R_f$ values (i.e., distance migrated by the visualized spot relative to the solvent front) for fluorescein and Fl-PE were 1.0 and 0.45, respectively. The Fl-PE band was scraped off the plates, eluted with CHCl₃/MeOH (2:1, vol/vol), taken to dryness under N₂ in a rotary evaporator, redissolved in 0.5 ml CHCl₃ and taken through the preparative thin layer chromatography procedure three more times. The product was analyzed by fluorescence in absorption spectrophotometry and for phospholipid phosphorus by the method of Raheja et al. (29). The ratio of fluorescein to phospholipid phosphorus in the Fl-PE was 0.9:1.0.

Preparation of Schistosomula and Adult Schistosomes

The life cycle of a Puerto Rican strain of $S. mansoni$ was maintained by passage through laboratory mice strains and Biomphalaria glabrata snails. Adult worms were recovered by perfusion of the hepatic portal system of infected mice (40), and kept in Eagle's medium (25) containing 10% fetal calf serum or newborn calf serum at 37°C. Experiments were carried out within 24 h of isolating the worms. Schistosomula were obtained after infection. In vitro, the parasites were then washed four times in Eagle's medium lacking calf serum, and either mounted directly for microscopic examination or first subjected to the vesiculation procedure below. When Fl-PE was the probe, it was added to 1.0 ml of Eagle's medium as 10 μg of Fl-PE in 5 μl of CHCl₃. The solution was then ultrasonicated for 5-10 s before adding the parasites, and the procedure then followed that used with the other fluorescent lipid probes. For microscopic examination, the labeled parasites were placed on a microscope slide in 80 μl of Eagle's medium containing 50 μg of the paralyzing agent carbamylecholine chloride (Sigma Chemical Co., St. Louis, MO) to prevent worm movement. The solution on the slide was confined by a ring or square of silicone grease, and finally sealed by addition of a coverslip.

Vesiculation Procedure

Membrane vesicles or blebs were prepared by applying the hypertonic saline procedure of Kusel et al. (24) to schistosomula and adults. Briefly, the procedure involves incubation of the parasite for 1 h at 4°C and then an additional 1 h at 25°C in 1.19 M NaCl in 25 mM Tris-HCl buffer, pH 7.4.

Fluorescence Microscopy and Fluorescence Photobleaching Recovery

Fluorescence photomicrographs were taken with a Leitz Ortholux II fluorescence microscope, a standard camera attachment, and Kodakolor II 200 ASA daylight film. Measurements of the two-dimensional lateral diffusion of fluorescent lipid probes incorporated into the membranes of parasites were made by the FPR method (2, 23) using the laser-microscope combination described by Garland and colleagues (16, 21, 30). In the latter part of the study we have satisfactorily used a low powered air-cooled argon ion laser (Lexel model 65; 100 mW all lines maximum power) instead of the more usual water-cooled laser of higher power (e.g., Lexel model 75; 1 W all lines maximum power). A 40X water-immersion objective lens was used throughout. The 1/e² radius of the focused laser spot at the sample was 1-2 μm. As calculated from measurements of the FPR by two-dimensional diffusion of fluorescent cellulose bovine serum albumin in thin films of glycerol of known viscosity (46). The characteristic times for recovery of fluorescence after photobleaching were calculated either by the simple 3-point method of Axelrod et al. (2), or by a nonlinear least squares fitting procedure as described by Bevington (3) to fit equation 12 of Axelrod et al. (2), which is the exact solution for the fluorescence recovery function in the case of a laser spot with a Gaussian transverse energy mode. When the latter procedure was used experimental data were collected with a Hewlett-Packard Model 85 microcomputer connected with the fluorescence measuring electronics (which for FPR measurements were analogue, not photon counting) by a Microlink interface (Biodata Ltd., Manchester, England). The data were stored on disk and subsequently transferred to a Dec 10 mainframe for running the curve-fitting program in Fortran.

Fluorescence Quenching

The depth of insertion of lipid probes in the membrane was determined by measuring the ability of a non-permeant molecule (trypan blue 0.25% w/v) to quench probe fluorescence by Förster resonance energy transfer. The principles of energy transfer theory have been reviewed by Stryer (43), and of depth dependent fluorescent quenching in micelles and membranes by Blatt and Sawyer (4). We calculated the $R_0$ value (i.e., the distance at which the energy transfer efficiency is 50%) from the absorption spectrum of trypan blue and the emission spectra of the lipid probes. This value was found to be between 3.8 and 4.2 nm for all the lipid probes used. The fluorescence intensity in these energy transfer experiments was measured with a photon-counting variation of the FPR apparatus (30). We also used an additional guard filter (3 mm of Schott BG 18 glass) to cut out the dull far red fluorescence emitted by trypan blue. We obtained independent experimental evidence of the calculated $R_0$ value in the case of fluorescein from a Stern-Volmer plot (4) of the fluorescence quenching of fluorescein-conjugated dextran (average molecular weight = 10,500) at varying concentrations of trypan blue. The value obtained was $5.0 \pm 1.0$ nm, and is in reasonable agreement with the theoretical value. Inner filter effects due to the absorption by trypan blue of either the excitation (544 nm) or emission (>550 nm) wavelengths were removed by measuring front face fluorescence in 100-μm (path length) microcuvettes (Camlab, Cambridge).

Electron Microscopy

Parasites were prepared for transmission electron microscopy by the method of Hockley and McLaren (19) except that schistosomula were fixed in glutaraldehyde for 3 h and adult worms for 4 h. Secondary fixation was for 2 h at 40°C in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.4.

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7.4. Thin sections were examined using a Phillips EM 300 electron microscope.

Results

General Form of the Experimental Data

To illustrate the form of FPR measurements, we show in Fig. 1 the data obtained from a single experimental measurement of $D_t$ for C$_{18}$-Fl labeling the surface of an adult male schistosome. Also shown is the computer-generated least squares best fit for a single value of $D_t$, along with the residuals for that fit and the autocorrelation function of the residuals. As is usual with such measurements, fluorescence was continuously monitored from the small area (radius 1.2 μm) illuminated by the focused (but greatly attenuated) laser beam at the surface under study, in this case the outer membrane of the tegument. After establishing a steady reading of the fluorescence signal, the average laser beam power was increased by $10^4$ for ~0.1 s before returning to the previous power. The high intensity pulse irreversibly bleached almost a half of the fluorescent molecules in the membrane at the focused laser spot. Fluorescence subsequently recovered due to unbleached molecules in the membrane beyond the laser spot diffusing laterally into the spot. $D_t$ was calculated from the kinetics of fluorescence recovery (2). In an idealized bilayer of the fluid mosaic model (37), $D_t$ would be expected to be $\sim 10^{-8}$ cm$^2$ s$^{-1}$ and recovery of fluorescence

Figure 1. FPR curve for C$_{18}$-Fl in the outer membrane of the body of an adult schistosome. In a, the observed fluorescence intensity has been plotted after normalization to a pre-bleach intensity of 1.0 arbitrary units. Channel 0 on the time axis corresponds to the end of the bleaching pulse. The channel width was 0.125 s. The smooth curve is the nonlinear least squares best fit to the data for a single diffusing species. In b, the residuals (i.e., the difference between the observed value and fitted value) have been plotted. A random scatter of the residuals and their (c) autocorrelation function around zero indicates a satisfactory fit of the calculated curve to the observations.
would ultimately go to completion (6). In the experiment shown, $D_0$ was $1.7 \times 10^{-9}$ cm$^2$ s$^{-1}$, which is relatively slow for a lipid probe.

The final extent ($R$) to which fluorescence recovery proceeds to complete recovery is given, in percentage terms, as $R = 100 \times \frac{(F_{oo} - F_o)}{(F_i - F_o)}$; where $F_{oo}$ is the fluorescence signal at infinite time after photobleaching, $F_o$ is the fluorescence signal immediately after photobleaching but before recovery, and $F_i$ is the fluorescence signal before photobleaching. In practice $F_{oo}$ is either taken as the fluorescence level beyond which no further recovery appears to occur or, better, by computer fitting of the data to a theoretical curve. In Fig. 1 the computed value for the percentage recovery was 85%, implying that 15% of the fluorescent molecules were unable to diffuse laterally (the so-called immobile fraction), at least on the time scale of the experiment and over the distance of 1 μm or so set by the radius of the laser spot.

**Studies with Adult Schistosomes**

Our earlier studies suggested that the surface properties of *S. mansoni* adults may vary with anatomical position on the worm (22). To examine this point further, we measured $D_0$ for the probe C18-diI at the head, ventral sucker, neck, and main body of the adult male worm. Fig. 2 shows an adult male and female pair with these anatomical regions labeled on the male worm. Table I summarizes the results. In all four regions studied $D_0$ was similar, in the range 3.2-5.8 $\times 10^{-9}$ cm$^2$ s$^{-1}$. The percentage recovery was only 20% in the body region, and highest at the ventral sucker. The gynaecophoric canal could not be studied because the surrounding worm body was not optically transparent. Nor could adult female worms be studied because, even when free or protruding from the gynaecophoral canal, their high level of autofluorescence interfered with detection of fluorescent probes.

The lateral diffusion of a range of other lipid probes was measured on the body surface of adult male schistosomes. The results are summarized in Table II. There were marked differences between different types of probes. The alkyl-substituted indocarbocyanines, C16-diI and C18-diI, exhibited low percentage recoveries (25-34%), as did Fl-PE. All the other probes had high percentage recoveries (>75%) as shown in Fig. 1 for C18-Fl. We were able to obtain adequate surface labeling with the longer chain length indocarbocyanines, C20-diI and C22-diI. Nevertheless, it is clear

![Figure 2. A pair of adult male (♂) and female (♀) *Schistosoma mansoni* as viewed by scanning electron microscopy. H, head; V, ventral sucker; G, gynaecophoric canal; D, dorsal surface of body; O, oral sucker. Bar, 250 μm.](image-url)
Table I. Lateral Diffusion* of C16-dil in the Outer Membrane of Adult Schistosomes

| Region      | $D_L (\times 10^{-9} \text{ cm}^2 \text{ s}^{-1})$ | % Recovery |
|-------------|-----------------------------------------------|------------|
| Ventral sucker | 4.7 ± 2.0 (22)                                | 62 ± 12    |
| Head        | 5.8 ± 1.8 (24)                                | 42 ± 10    |
| Neck        | 4.9 ± 2.3 (21)                                | 40 ± 10    |
| Body        | 3.2 ± 2.0 (23)                                | 20 ± 9     |

* The values of $D_L$ and % recovery are given as mean ± standard deviation, with the number of measurements in parentheses. Approximately 3–6 measurements were made on each region for any one worm.

from the data of Table II that the length of the alkyl chains in the probes did not determine whether a probe fell into the group with low (Fl-PE, C16-dil, C18-dil) or high (C16-FI, C15-FI, C18-Rh) recovery. Statistical analysis was done by the Student’s t test and differences described in the text are significant ($p < 0.001$). $D_L$ values for the mobile fractions of probes were in the range of 3.1 to $4.8 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$, with the exceptions of C18-Rh and Fl-PE which had $D_L$ values some 10-fold lower. All of the results presented so far refer to worms at 35–37°C. No effects of lower temperature down to 6°C were found, other than a two- to threefold diminution of the $D_L$ value. Percentage recoveries were unaffected at the lower temperatures. The $D_L$ values are rather low for all probes and C16-dil and Fl-PE have unusually low recoveries when compared to lipid analogues in bilayers (6, 14). Fluorescence microscopy of the worms labeled with the different probes in all cases revealed the same pattern of body surface labeling, similar to that described (22) for fluorescent wheat germ agglutinin or concanavalin A which are presumably non-permeant. It therefore seems very likely that the lipid probes have indeed labeled the surface membrane and have not labeled other membranes. Neither long chain alkyl fluoresceins nor carbocyanines fluoresce when they form micelles (36, 38). It therefore follows that the fluorescence which we observe does not arise from micelles of the probe adsorbed to the membrane surfaces.

The Effect of Bleb Formation on Lipid Probe Diffusion

After incubating parasites with the lipid probes, membrane blebs were formed from parasites by hypertonic saline treatment. Peripheral fluorescent staining was observed in blebs with all lipid probes used (listed in Table II) as was the case with several fluorescently labeled lectins (namely, rhodamine concanavalin A, rhodamine wheat germ agglutinin, and fluorescein peanut agglutinin). Bleb formation did not significantly alter the $D_L$ value of lipid probes: it remained at $\sim 2-6 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$, an order of magnitude lower than the values usually obtained for eucaryotic cell membranes. However, the division of the lipid probes into a group with low recovery and a group of high recovery found in the normal outer membrane (Table II) was abolished in the outer membrane of blebs: all exhibited a high recovery of >80%. Table III summarizes such results with C16-dil and C16-FI as representative of the low and high recovery groups, respectively.

| Lipid       | $D_L (\times 10^{-9} \text{ cm}^2 \text{ s}^{-1})$ | % Recovery |
|-------------|-----------------------------------------------|------------|
| Fl-PE       | 0.42 ± 0.09 (11)                             | 29 ± 10    |
| C16-dil     | 3.1 ± 2.4 (16)                                | 25 ± 15    |
| C16-dil     | 4.0 ± 1.0 (68)                                | 34 ± 12    |
| C16-FI      | 3.5 ± 2.0 (24)                                | 76 ± 8     |
| C18-FI      | 4.8 ± 1.6 (30)                                | 75 ± 10    |
| C18-FI      | 3.6 ± 1.9 (32)                                | 79 ± 19    |
| C18-Rh      | 0.40 ± 0.07 (10)                              | 79 ± 15    |

* The values of $D_L$ and % recovery are given as mean ± standard deviation, with the number of measurements in parentheses. Approximately 3–4 measurements were made on any one worm.

Table II. Lateral Diffusion* of Various Fluorescent Lipids in the Outer Membrane of the Body of Adult Schistosomes

Table III. Effect of Bleb Formation on the Lateral Diffusion* of C16-FI and C16-dil in the Outer Membrane of the Body of Adult Schistosomes

| Lipid     | State of membrane | $D_L (\times 10^{-9} \text{ cm}^2 \text{ s}^{-1})$ | % Recovery |
|-----------|-------------------|-----------------------------------------------|------------|
| C16-dil   | Normal            | 3.2 ± 2.0 (23)                               | 20 ± 9     |
| C18-dil   | Bleb              | 3.9 ± 0.8 (21)                               | 83 ± 11    |
| C16-FI    | Normal            | 3.6 ± 1.9 (32)                               | 79 ± 19    |
| C18-FI    | Bleb              | 2.3 ± 0.5 (19)                               | 84 ± 8     |

* The values of $D_L$ and % recovery are given as mean ± standard deviation, with the number of measurements in parentheses. Approximately 4–6 measurements were made on any one worm, and were restricted to the body region or blebs attached to the body region.

Structure of Outer Membrane Blebs

Studied by Light Microscopy

The formation of blebs and vesicles at the surface of schistosomes in response to hypertonic saline treatment (24) can readily be followed by phase-contrast microscopy or, if the outer membrane has previously been stained with a fluorescent lipid probe, by fluorescence microscopy. Fig. 3 shows micrographs of views by phase-contrast (Fig. 3, A and B) and fluorescence microscopy (Fig. 3 C) of schistosomes labeled with C16-dil. Initially blebs or blisters form (Fig. 3 A) and progress with time to attached (Fig. 3, B and C) and then free vesicles. The ring-like distribution of fluorescence in the vesicles shows that it is the surface of the vesicle that is labeled, not the interior. The highly patterned fluorescent staining characteristic of the normal adult surface was lost in the vesicles and blebs. These observations are similar to those previously observed with schistosomula (24) and also to those obtained using C16-FI in place of C18-dil (data not shown).

Structure of Outer Membrane Blebs

Studied by Electron Microscopy

Electron micrographs of thin sections through blebs formed on the surface of adult schistosomes showed outer membrane carrying, attached to its inner aspect, many membranous structures and small vesicles (Fig. 4 D). The highly indented and pitted surface of the normal adult became smoothed out and flattened in the vesicles, in keeping with the loss of surface pattern seen by fluorescence microscopy. It appears that the vesicles were formed by detachment of the tegument, carrying with them the basal membrane (Fig. 4 C). Thus the vesicles and blebs are bounded by a complex layer, and not just a bilayer as in the blebs described by Tank et al. on different organisms and cells (44).

We also studied by electron microscopy vesicles formed by hypertonic saline treatment on the surface of the schistoso-
Figure 3. Light microscopy of blebs induced by hypertonic saline treatment of adult schistosomes. A and B are phase-contrast micrographs and C is a fluorescence micrograph. The worms had been stained with C<sub>3</sub>-dil before hypertonic saline treatment. Bars, 20 μm.
Figure 4. Electron micrographs of the outer region of adult schistosomes before and after bleb formation induced by hypertonic saline. (A) The normal tegument is bounded by a basal membrane (bm) internally and a surface membrane (sm) externally. m, muscle; p, pit. (B–D) Progressive stages in bleb formation. sv, small vesicles. Bars, (A, C, and D) 0.5 μm; (B) 2 μm.

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mula. They differed from those seen in the adult; separation of the vesicle appeared to leave the basal membrane of the tegument still attached to the body of the worm, and consequently the outer layer of the vesicle was derived from the double outer membrane of the tegument with less membrane structure attached to the inner surface (Fig. 5, A and B). It appeared that the double outer bilayer structure remained intact in the blebs. (Fig. 5 C).

**Vertical Distribution of Fluorescent Lipids in the Outer Membrane**

A lipid analogue incorporated into the outer membrane (which, as described above, has two bilayers) could be situated in any one or more of the four constituent monolayers (i.e., the outer and inner halves of the outer bilayer, and the outer and inner halves of the inner bilayer). To explore these possible locations we used trypan blue as a non-permeant quencher of fluorescein (or rhodamine and di-indocarbocyanine) fluorescence on the external (outer) aspect of the membrane. The absorption spectrum of trypan blue overlaps the emission spectra of the fluorescent lipids used, and the mechanism of quenching is most probably by Forster resonance energy transfer. We calculated that a trypan blue molecule would have a 50% probability of accepting energy if it were 3.8-4.2 nm from an excited fluorescent donor. Because the fluorophores are hydrophilic, they will be located at the head group regions of the bilayers. The possible locations are shown schematically in Fig. 6. The relationship between efficiency of energy transfer and increasing separation of donor and acceptor is an inverse sixth power law. Accordingly, only those fluorescent lipids with headgroups on the external surface of the outer membrane could be quenched by a non-permeant energy acceptor such as trypan blue; fluorophores located in any of the other headgroup regions are too far removed from trypan blue to be significantly quenched.

Quenching data are summarized in Table IV. In the normal parasite \( C_{II} \)-Fl and \( C_{II} \)-Rh are both severely quenched by trypan blue, which would indicate that both lipids are located mainly in the outer monolayer of the outer bilayer. \( C_{II} \)-diII, however, is deduced to reside in one or more of the remaining three monolayers, since its fluorescence was only slightly quenched. On average, just less than half of the FI-PE molecules reside in the outer monolayer of the outer bilayer as judged by this method.

The formation of membrane blebs alters the vertical distribution of the lipids in the adult schistosome membrane, since the data show that all fluorescent lipids in blebs are extensively quenched by trypan blue, from which we conclude that the fluorescent lipids appear to be located in the outer monolayer of the outer bilayer. These data also indicate that the fluorescent lipid is not gaining access to the membranous structures and small vesicles observed within adult blebs. (Fig. 4).

**Studies with Schistosomula and Lung Forms**

Lateral diffusion of two fluorescent lipids, \( C_{II} \)-diII and \( C_{II} \)-Fl was examined on the double outer membrane of schistosomula and lung worms. The results are described in Table V. The data illustrate that the \( D_{L} \) values of both lipids are \( \sim 10 \)-fold lower than for the adult membrane. It is also noted that unlike adults, both schistosomula and lung worms show a restricted diffusion of \( C_{II} \)-Fl. Both skin and mechanically transformed schistosomula gave very similar FPR results. Quenching experiments reveal that the majority of the fluorescent lipid molecules for both probes are incorporated into the outer monolayer of the outer bilayer (data not shown). Thus it appears that in both schistosomula and lung worms as distinct from adults there is a different vertical distribution of fluorescent lipids in the surface membrane.
Table V. Lateral Diffusion of Two Fluorescent Lipids in the Outer Membrane of Schistosomula and Lung Worms

| Lipid          | Developmental stage | $D_c$ ($\times 10^{-9}$ cm² s⁻¹) | % Recovery |
|----------------|---------------------|---------------------------------|------------|
| C₁₀-diI        | Schistosomula        | 0.30 ± 0.10 (17)                | 30 ± 13    |
| C₁₄-diI        | Schistosomula        | 0.33 ± 0.13 (10)                | 61 ± 16    |
| C₁₄-fluor       | Schistosomula        | 0.27 ± 0.06 (17)                | 55 ± 11    |
| C₁₆-fluor      | Schistosomula        | 0.33 ± 0.13 (10)                | 61 ± 16    |

* Schistosomula transformed by the mechanical process were examined between 6 and 24 h after transformation from cercaria when the double outer membrane should be present. Lung worms were used between 2 and 24 h after perfusion from the lungs of infected mice.

Discussion

FPR and Energy Transfer Studies of the Schistosome Membrane

Using the technique of FPR it has been possible to measure the lateral diffusion of various fluorescent lipids inserted into the outer membrane of S. mansoni. We have assumed throughout this work, as have many others in this field, that the lipid probes intercalate into the surface lipid bilayers. We can exclude from our observations surface aggregates or micelles of the probe because such assemblies do not fluoresce (36, 38). Furthermore, surface adsorption of probes by mechanisms other than bilayer intercalation (e.g., electrostatic interactions with phospholipid head groups) do not necessarily lead to low $D_c$ or percent recovery values. An additional point is that the schistosomula surface is known to take up host globulins, and this has been modeled using transfer of carboxcyanin dyes from erythrocyte ghosts (5). As revealed in Fig. 2, the surface of this parasite has a rather complex topography and the membranes cannot be regarded as simply planar bilayers. Although an unusual effect on lateral lipid diffusion due to surface convolutions cannot be ruled out, several studies both experimental and theoretical, demonstrated only small effects of non-planar topography on lateral diffusion of lipid probes (1, 13, 49). It is probably the case that the apparent $D_c$ values obtained in our study will be reduced by only two- to threefold; however, the observed differences between lipid analogues will still be valid and the fractional recoveries unaffected.

One unusual finding is that three fluorescent lipids, C₁₀-diI, C₁₄-diI, and Fl-PE give very low mobile fractions whereas the others did not. In most other biological systems, lipid probes such as C₁₀-diI behave as in pure lipid bilayers giving a mobile fraction of almost 100% and a high $D_c$ value of $\sim 10^{-9}$ to $10^{-8}$ cm² s⁻¹. Thus there is restricted lipid diffusion in the outer membrane of adult S. mansoni. Lipid domains as detected by restricted diffusion of fluorescent lipids have been observed by other workers (10, 50). Quenching of incorporated lipid fluorescence was used to determine the vertical distribution of several fluorescent lipids partitioned into the membrane. Our results revealed that the majority of molecules of those lipids which were not restricted in their lateral diffusion (i.e., C₁₄-Fl and C₁₈-Rh) were located in the outer monolayer of the outer bilayer. However, the lipids that exhibited restricted lateral diffusion (i.e., C₁₈-diI and Fl-PE) were found to reside mainly in one or more of the remaining monolayers of the adult membrane.

Differing lipid distribution across membranes has been described in other biological systems (34, 35), including the observation that phosphatidylethanolamine is largely within the inner leaflet of cell membranes. Our results are in general agreement with Dragsten et al. (11, 12) who, in a study using fluorescent lipids on epithelial cells, concluded that 1,1'-di-(hexadecyl)-3,3',3'-tetramethylindocarbo cyanine perchlorate (C₁₈-diI) can flip-flop and locate itself in the inner monolayer of those cells whereas C₁₈-Fl cannot.

Although the presence of one or two hydrophobic chains on the lipid probes appears to correlate with high or low percent recovery, respectively, and might lead one to propose that the single-chained lipids do not undergo transmembrane flip-flop whereas the double-chained lipids do, we think this is unlikely unless the chain-length range is restricted to C₁₈ upwards. This is because 5-N-(dodecanoyl)-aminoindocarbocyanine (C₁₂-Fl) could label only the outer surfaces but also the body interior when applied externally, showing that it could cross many membranes (data not shown), whereas none of the other probes labeled beyond the outer surface.

The production of membrane blebs from adult worms has allowed the study of fluorescent lipid diffusion on the outer membrane detached from the rest of the worm body. This technique of bleb formation has been used in other studies to correlate enhanced protein lateral diffusion in the membrane bleb with the disruption of an underlying cytoskeletal network (44). Although cytoskeletal constraints have not been invoked to explain the much rarer phenomenon of restricted lipid diffusion in plasma membranes, we did observe an analogous release of restrictions of lipid diffusion on adult blebs. This increase in the fraction of molecules that can diffuse laterally does not seem to occur simply as a result of the reduction of pits and folds of the adult membrane since Wolf et al. found only slight differences in the diffusion of a lipid probe due to the effect of surface microvilli (49). In addition studies have inferred a physical connection between lipids in plasma membrane and the cytoskeleton (31, 47). The differing distribution of fluorescent lipids found in the normal adult outer membrane was altered by bleb formation as observed by fluorescence quenching experiments, in which all four fluorescent lipids used were extensively quenched when in membrane blebs. The differences, described in this study, between the lateral diffusion of fluorescent lipids on juvenile stages of the parasite and mature adult worms, indicate that as the parasite develops a major change in membrane structure or organization occurs. This has been suggested previously by a number of workers from independent methods (25, 26). Fluorescence quenching studies on schistosomula and lung worms indicates that most of the molecules of all the fluorescent lipids examined are in the outer monolayer of the outer bilayer. Since restricted lipid diffusion can be observed in these stages it would suggest that there are gel phase and lipid crystalline phases in this external monolayer. Hence there is horizontal lipid asymmetry in this monolayer in the juvenile stages of the parasite as opposed to vertical asymmetry in the adult.

Membrane Organization

Extensive tetralayers consisting of two bilayers tightly apposed face-to-face over a large area are rare in nature, and have probably not been studied previously with the methods used by us. Consequently, we have initially interpreted our
results in terms of the more familiar properties of bilayer membranes. Our interpretation of these results for the adult surface indicates that the outer monolayer of the outer bilayer allows the greatest freedom for a lipid to diffuse laterally (i.e., it behaves as a typical biological membrane with respect to lipid lateral diffusion). One or more of the remaining three monolayers however restricts lipid diffusion drastically, and in this sense is atypical of most biological membranes. Because the observed restricted lipid diffusion is diminished on membrane blebs, it is tempting to speculate that it is the parasite cytoskeleton which maintains this lipid restriction in the normal adult. Studies involving the lateral diffusion of membrane proteins on adult worms and blebs may clarify this issue.

Biological Significance

It is possible that the observed immobility of lipid molecules in the outer membrane of adult schistosomes may play an important role in allowing the parasite to resist immune attack. For example, there is a considerable body of evidence which suggests that in order for complement to be fixed and the lesion-forming membrane attack complex to integrate into the membranes rapid lateral diffusion is required in the membrane. Thus restricted lipid diffusion may be the mechanism used by schistosomes to survive host immune attack, although the relatively "fluid" external monolayer would presumably provide a suitable environment for complement fixation.

Restricted lipid diffusion may have a role to play in allowing the schistosomula and the lung worms to evade the immune response; if this is so then the restriction on lipids in the young stages differs from that in adults. The outer bilayer behaves as if it has both gel and liquid crystalline phase domains (data not shown). This may allow less immune damage to the parasite than an external monolayer which was solely in the liquid crystalline phase by, for example, preventing lateral diffusion of antigens in the juvenile parasite surface. If this idea of lipid immobility being involved in evasion of the immune response is accurate, then it may follow that parasite membrane blebs, with no observed lipid immobility, should be susceptible to host immune damage. We are currently investigating this possibility.

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