Uptake and compartmentalization of fluorescent lipid analogs in larval *Schistosoma mansoni*

Stephen T. Furlong,[1,*,†] Kathleen S. Thibault,[†] Laetitia M. Morbelli,[†] John J. Quinn,[**] and Rick A. Rogers[††]

Department of Medicine,* Harvard Medical School, Department of Rheumatology/Immunology,† Brigham and Women's Hospital, and Departments of Tropical Public Health** and Environmental Health,†† Harvard School of Public Health, Seeley G. Mudd Building, 250 Longwood Avenue, Boston, MA 02115

Abstract Recent studies have suggested that host lipids are both a requirement for the human parasite *Schistosoma mansoni* and may play a role in evasion of host immunity. To study lipid utilization by this organism we have followed the uptake of fluorescent fatty acid and phospholipid analogs in two parasite stages, cercariae and schistosomula. As determined by both morphological and biochemical methods, a fluorescent fatty acid analog labeled with bodipy was incorporated into both stages. In cercariae, diffuse fluorescence was present throughout the organism and discrete lipid droplets were observed in the tail and in the anterior structures. In contrast, fluorescence distribution in cercariae transformed to schistosomula was restricted to cytoplasmic lipid droplets throughout the organism. Biochemical analysis demonstrated that the fatty acid analog was biosynthetically incorporated primarily into neutral lipids but also somewhat into phospholipids. The percentage of free label decreased with time. Similar results were obtained when organisms were labeled directly in vitro or indirectly by labeling the intermediate snail host. Compared to the fatty acid analogs, localization of fluorescent phospholipid analogs by schistosomula was considerably different. Phosphatidylcholine labeled on short acyl chains with either bodipy or NBD localized primarily to a network of cells beneath the organism's surface. A longer chain bodipy-labeled phosphatidylcholine localized to the parasite surface, gut and acetabulum. These studies show specificity in the transport of lipid analogs by this important human parasite, elucidate the compartments within the organism in which specific lipids preferentially accumulate, and demonstrate stage-dependent differences in the utilization of exogenous lipids by this organism.—**Furlong, S. T., K. S. Thibault, L. M. Morbelli, J. J. Quinn, and R. A. Rogers.** Uptake and compartmentalization of fluorescent lipid analogs in larval *Schistosoma mansoni*. *J. Lipid Res.* 1995. **36:** 1–12.

Supplementary key words fluorescent fatty acid • phosphatidylcholine • fluorescence microscopy • confocal microscopy

It is estimated that more than 200 million people worldwide are infected with one of several *Schistosoma* species. Such infections contribute to nearly a million deaths annually. Although drugs are available to treat schistosomiasis, difficulties exist in distribution of the drug in third world countries and re-infection in endemic areas is a problem (1). Thus, the focus of much schistosome research is vaccine development. Unfortunately, high levels of protection are difficult to achieve in animal models and, as yet, no successful vaccine has been developed for use in humans (2). Recent evidence has suggested that some clues to the parasite's evasion of host immunity may be provided by understanding its lipid biochemistry. Specifically, schistosomes cannot make fatty acids or sterols de novo (3, 4) and thus must acquire host lipids. Host lipoproteins bind to the parasite's surface and host immune cells lyse probably due to lysophosphatidylcholine (5–7). Either or both of these phenomena may provide a host lipid source for the parasite as well as protection from host immunity. However, there is little direct evidence to illustrate how the parasite utilizes such host lipids. Such information is important for understanding the parasite's biology and may be of practical significance for vaccine development.

The life cycle of *Schistosoma mansoni* is complicated. This parasite has multiple developmental stages and two hosts, snails and humans. Cercariae are the free-swimming stage of *S. mansoni* which is released from snails that subsequently infect humans. Following invasion of human skin, cercariae rapidly transform to schistosomula. During this transformation, the parasite undergoes both morphological and biochemical changes that adapt it to the host environment. These changes include biogenesis of a double lipid bilayer that completely surrounds the organism and loss of the cercarial tail and glycocalyx. Fluorescent fatty acid and phospholipid analogs provide the opportunity to follow incorporation of these lipids both

Abbreviations: ddH_2O, double-distilled H_2O; SPE, solid phase extraction; HPLC, high performance liquid chromatography; CLSM, confocal laser scanning microscope; DAB, diaminobenzidine.

[1]To whom correspondence should be addressed.

biochemically and morphologically (8) during these two important stages in the parasite's life cycle and during the transformation process itself. The goals of these studies were to follow uptake of fatty acids and phospholipids by the parasite, to elucidate the compartments within the organism where the lipids accumulate, and to examine how lipid structure influences localization. In addition, we also wished to determine whether the developmental stage of the parasite influenced lipid distribution. Results from this study show clear differences in processing of lipids by the parasite based on lipid structure and on stage of the organism's development. Furthermore, these studies demonstrate that *S. mansoni* represents a useful model system for examining lipid transport in a multicellular organism.

MATERIALS AND METHODS

Preparation of cercariae and schistosomula from *Schistosoma mansoni*

Cercariae (Puerto Rican strain) were obtained from infected snails (*Biomphalaria glabrata*) by shedding. Schistosomula were mechanically prepared from cercariae by vortexing (9) and the resulting bodies and tails were separated on a Percoll density gradient. The bodies were washed three times and resuspended in RPMI 1640 at 37°C for 3 h to allow complete transformation (10). Schistosomula were cultured at a concentration of 1×10^3 organisms/ml in RPMI 1640 containing 0.1% BSA, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C under an atmosphere of 5% CO_2. Viability, as assessed by examining the cultured organisms for head and torso movement and flame cell activity, was greater than 95% for all experiments. Prior to labeling, the worms were washed three times in sterile RPMI (void of any supplements).

Labeling of cercariae and schistosomula with fluorescent lipid probes

Cercariae were labeled with a fatty acid analog 4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-3-indacene-dodecanoic acid (Bodipy-C_{12}) (Molecular Probes, Eugene, OR). Schistosomula were labeled with Bodipy-C_{12} or one of three phosphatidylcholine analogs; 2-(4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-3-indacenepentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (C_5-Bodipy-PC), 2-(4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-3-indacenedodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (C_{12}-Bodipy-PC) and 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)-amino]caproyl] phosphatidylcholine (C_6-NBD-PC). Freshly shed cercariae were labeled by addition of 2 μl of 100 μM Bodipy-C_{12} in DMSO to 1 ml of cercariae in water. To label schistosomula after transformation, worms were harvested and washed three times with fresh, sterile RPMI (void of any supplements). After the final wash the worms were divided into 10,000 organism portions for labeling. For Bodipy-C_{12} short-term labeling experiments, worms were incubated at 37°C for 15 min to 2 h in 1 ml of RPMI containing 40 nM, 100 nM, 200 nM, 400 nM, 1 μM, and 2 μM Bodipy-C_{12} (final concentrations). Aliquots of organisms from each concentration were analyzed biochemically. The optimum concentrations for photomicrography were determined empirically in initial experiments as 200 nM for short-term labeling experiments or 2 μM for labeling followed by overnight incubation. These concentrations were used for all subsequent morphological evaluations. Bodipy-C_{12}-labeled worms were viewed and photographed using the H_2 or N_2 cube with either a Leitz orthoplan or Leitz ortholux microscope. To determine the temperature dependence of lipid uptake, parallel experiments for lipid analogs were carried out at 0°C and 37°C.

To label cercariae with fatty acid analog prior to release from infected snails, five to ten patent *Biomphalaria glabrata* snails, 4 weeks post-infection with 7–10 miracidia of *S. mansoni,* were rinsed in double-distilled H_2O (ddH_2O) and incubated without light for 24 h in water containing the fatty acid analog. The labeling solution was prepared by dissolving Bodipy-C_{12} in DMSO to a concentration of 1 mM and adding 50 μl of this solution to 50 ml ddH_2O. An equal number of control snails were incubated with DMSO as above, but without Bodipy-C_{12}. After the 24-h labeling period, the snails were rinsed copiously with ddH_2O and placed in dark, aerated aquaria containing 6 liters of ddH_2O for an additional 24 h. At the end of the experiment, snail viability was 93% for control snails (n = 24) compared with 90% for labeled snails (n = 25). The difference in viability from three separate experiments was not statistically significant. Free-swimming cercariae were collected from the Bodipy-C_{12}-labeled and control snails after 1.5 h exposure to light as described (10). Bodipy-C_{12}-labeled cercariae were either examined immediately by epifluorescence microscopy or transformed, cultured in RPMI, and examined at intervals from 3 to 24 h post-transformation. Fatty acid-labeled snails were returned to the dark and shed on days 2, 7, 14, and 21 post-label to determine the persistence of the label. Cercariae from both groups of snails were determined to be viable from swimming activity and flame cell beat. No difference in cercarial emergence was observed between groups. However, for photography, 10^{-2} M eserine sulfate (Sigma Chemical Co., St. Louis, MO) was added (giving a final concentration of 10^{-3} M) to paralyze the cercarial movement (11). Schistosome larvae were also isolated from the hepatopancreas of labeled or control snails and examined by fluorescence microscopy as above. Briefly, three snails from each group were gently crushed in a glass petri dish. The snail body was teased away from the shell and the hepatopancreas was isolated. The hepatopancreas was teased apart with 25-gauge needles and the larvae were collected and washed twice in ddH_2O containing 10^{-3} M eserine sulfate.

Labeling of schistosomula with fluorescent phospholipids

Schistosomula were labeled with phospholipid analogs by incubation with analog-containing liposomes. Liposomes were prepared by ethanol injection as previously described (8). In brief, 2 mg of C_6-NBD-PC stock solution plus 3 mg egg PC were dried from chloroform under nitrogen in a glass centrifuge tube. The dried lipid was resuspended in 100 μl of 100% ethanol and 35 μl of this solution was injected into 10 ml sterile RPMI (final concentration 90 μM relative to NBD-PC, 230 μM total PC concentration). Liposomes containing C_5-bodipy-PC or C_{12}-bodipy-PC were prepared identically except that stock solutions were prepared from only 1 rather than 2 mg of fluorescent analog. For back exchange, liposomes were prepared with egg PC alone and no fluorescent phospholipid. Twenty mg of egg-PC was dried from chloroform under nitrogen and resuspended in 500 μl of 100% ethanol. Thirty-five μl of this solution was injected into 10 ml of fresh, sterile RPMI. Liposome preparations were prepared fresh for all experiments.

Extraction, separation, and quantitation of labeled lipids

For biochemical analysis, fluorescently labeled lipids were extracted from schistosomula with chloroform–methanol (12) and separated using either solid phase extraction columns or by HPLC. For solid phase extraction (SPE), samples were resuspended in chloroform, applied to 500 mg aminopropyl columns on a vacuum manifold, and separated into eight fractions. Lipid classes were identified based on eluting solvents exactly as described (13). Collected fractions were dried under nitrogen, resuspended in 100 μl of chloroform, and fluorescence was measured in a Perkin-Elmer spectrofluorimeter at excitation and emission wavelengths of 488 nm and 519 nm, respectively. To confirm the identity of the lipid fractions separated on the SPE columns, unlabeled standards were separated under identical conditions and the collected fractions were monitored by thin-layer chromatography (13).

To characterize phospholipid classes, labeled samples were separated by high performance liquid chromatography (HPLC) on a silicic acid column with a Waters automated gradient HPLC system on a 25 cm by 4.0 mm steel column packed with 5 μm Econosphere silica (Alltech Applied Sciences, State College, PA) with a mobile phase of acetonitrile–methanol–85% phosphoric acid 130:6:1.5 flowing at either 0.9 or 1.25 ml/min (5, 14, 15). Fluorescent lipids were detected with a Kratos 950 fluorescence detector with a 389 nm excitation filter and 500 nm emission filter. Lipids were identified by comparison to the retention times of unlabeled standards detected by a Waters model 441 UV detector at 214 nm and fluorescently labeled standards were detected with the fluorescence detector. Standards were purchased from Avanti Polar Lipids (Birmingham, AL). Data were collected with either a Nelson Model 760 or 960 interface and integrated with Nelson Analytical Software on a Hewlett-Packard Vectra computer.

Confocal microscopy

For confocal microscopy either a Bio-Rad MRC-600 or Sarastro 2000 Confocal Laser Scanning Microscope (CLSM) was used. Operating conditions for the Bio-Rad system were described previously (8). The Sarastro CLSM was fitted with a 25 mW argon-ion laser and instrument settings were: 488 nm excitation, > 535 nm emission, 10% transmission, laser power 15.9 mW, with a PMT voltage of 590 mV. Optical sections, 0.5-μm thick were collected with a 60× (1.4 numeric aperture) objective in the midline plane of every worm. To minimize sample bias, worms that exhibited slight head or torso movement or flame cell activity were chosen at random under bright field, then images were collected using fluorescent CLSM without previous examination with epifluorescent light.

Three-dimensional reconstruction

For three-dimensional reconstruction, serial optical sections were collected by CLSM as previously described (8) as above except the output of the laser was reduced to 10% transmittance, and each section was scanned only once. The Z-axis interval between each optical section was 0.9 μm. Typically, 25 optical serial sections were collected to completely scan an organism. For some studies 3-D images were also prepared using Bio-Rad confocal microscope images. For these reconstructions serial optical images were photographed directly from a flat screen monitor with a 35-mm camera. Images of serial optical sections were then projected onto a digitizing tablet from a ceiling-mounted Bessler point source enlarger and traced manually. Surface contour profiles of the outer tegumental membrane, fluorescent-labeled areas, and nuclei within labeled areas were entered via the digitizing tablet into HVEM 3-D, a 3-D reconstruction program (16). Section registration was corrected from the projected images by selection of fiducial points that spanned at least two adjacent sections. The reconstructed schistosomula was oriented about the X, Y, Z axis, and photographed directly from the computer monitor.

Electron microscopy

To correlate the fluorescent microscope localizations with ultrastructural information, schistosomula containing fluorescent NBD-PC were processed following the method of Pagano, Sepanski, and Martin (17) with modifications. It has been shown that intense fluorophore emission in the presence of 3-3' diaminobenzidine will result in the formation of an insoluble complex at the anatomic site of fluorophore deposition thus permitting the

same structures observed by fluorescence microscopy to be examined by electron microscopy (17). To improve resolution, living schistosomula were examined by fluorescent CLSM, then fixed in 1.5% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.4, on ice for 30 min. Worms were then washed 5 min in 0.1 M Na-cacodylate buffer, pH 7.4, and 0.1 M Tris-HCl, pH 7.6, containing 0.3% sodium azide, respectively, before being suspended in 0.1 M Tris-HCl, pH 7.6, containing 1.5 mg/ml diaminobenzidine (DAB) with 0.3% sodium azide added. Free worms were concentrated by centrifugation for 5 min at 400 *g* between washes. After the addition of DAB, the worms were transferred to depression slides and photobleached. The 25 mW argon-ion laser (100% transmittance) from the Sarastro CLSM was used to illuminate the worms with 488 nm light for 15 min. The slide was constantly moved as the laser light was passed through the worm suspension using a 4 × objective. Fresh DAB solution was added at 5-min intervals. Worms were then removed, washed in 0.1 M Tris buffer, pH 7.6, containing 0.3% sodium azide and 0.1 M Na-cacodylate buffer, pH 7.4, respectively, then post-fixed 60 min in 1% osmium tetroxide in 0.1 M Na-cacodylate buffer, pH 7.4, on ice. Pelleted worms were then washed in 0.1 M Na-cacodylate buffer, pH 7.4, and dehydrated in an ethanolic series to absolute. After infiltration in 1:1 propylene oxide–Spurr overnight, worms were embedded in Spurr (Ernest Fullam, Latham, NY) plastic and polymerized. Thin sections were cut and stained with lead and uranyl salts prior to examination in a Philips EM 300 transmission electron microscope.

RESULTS

The goals of these studies were: *1)* to label cercariae and schistosomula of *S. mansoni* with fluorescent fatty acid and phospholipid analogs; *2)* to localize these probes morphologically by fluorescence microscopy; and *3)* to characterize the incorporation of the fatty acid analog into the organism's lipids biochemically. In particular, we wished to determine whether the analogs were localized preferentially within the parasite, to correlate such localization with chemical structure of the lipid analogs, and to determine whether alterations in the distribution or transport of the fatty acid analog occur during transformation from cercaria to schistosomulum of this important human parasite.

In vitro labeling of cercariae and schistosomula with fatty acid analogs

Bodipy-C_{12} was readily incorporated by cercariae and schistosomula in vitro and labeling was time, concentration, and temperature dependent. The optimum labeling conditions for following incorporation of the analog into schistosome structures by microscopy was found to be 200 nM for 1 h for both cercariae and schistosomula. For cercariae, yellow-gold fluorescence was distributed diffusely throughout the body of the worm (**Fig. 1A**). Internalized lipid droplets were identified in the anterior portion of the worm, immediately beneath the tegument, and in the central regions of the tail. Labeling was less intense in the preacetabular glands whereas more of the lipid probe was present adjacent to the junction between body and tail. Bodipy-C_{12} labeling of cercariae was similar when examined for red fluorescence (Fig. 1B). However, less of the diffuse labeling was evident. Labeling in the preacetabular glands was absent and localization of lipid droplets was more pronounced. Red fluorescence observed in these samples undoubtedly reflects eximer emission due to high concentrations of the label in these samples (18). Thus, these results indicate that the high concentrations of Bodipy-C_{12} were found in the droplets.

To follow transformation-related changes in analog distribution as they occurred, cercariae were labeled with Bodipy-C_{12}, mechanically transformed, and observed at various intervals after the initiation of transformation. By 3 h post-transformation, differences were observed between labeled cercariae (Fig. 1A) and newly transformed schistosomula (Fig. 1C). In the newly transformed schistosomula, labeling was distributed in droplets throughout the organism and occasionally in the areas corresponding to the glands where labeling was previously absent. In samples labeled then cultured an additional 24 h (Fig. 1D), evidence of a less random droplet distribution was observed. Under these conditions label appeared in subtegumental structures. However, in no case, either immediately after transformation or 24 h post-transformation, was a significant amount of label found at the surface. When schistosomula were labeled with Bodipy-C_{12} after transformation, diffuse labeling similar to that observed in cercariae was observed (Fig. 1E). Similarly, after extended culture, labeling of lipid droplets was more pronounced (Fig. 1F). For either cercaria or schistosomula incubated on ice with the analog, labeling was absent indicating that incorporation of the probe is temperature dependent (data not shown).

To provide a higher resolution view of Bodipy-C_{12}-labeled structures within schistosomula, labeled organisms that had been cultured overnight were also examined by confocal microscopy. As shown in **Fig. 2A–C**, these experiments confirmed that the majority of the label was localized to intracellular lipid droplets and that little or no labeling was associated with the tegumental surface. However, some of the label was associated with subtegumental cellular membranes (arrow) suggesting incorporation into phospholipids (Fig. 2B). Some of the labeled cellular membranes included cells composing the organism's acetabulum (large arrow) (Fig. 2C). Nuclear staining of cells was not evident in any of the optical sections. However, some diffuse labeling was observed associated with subtegumental muscle bundles (Fig. 2A).

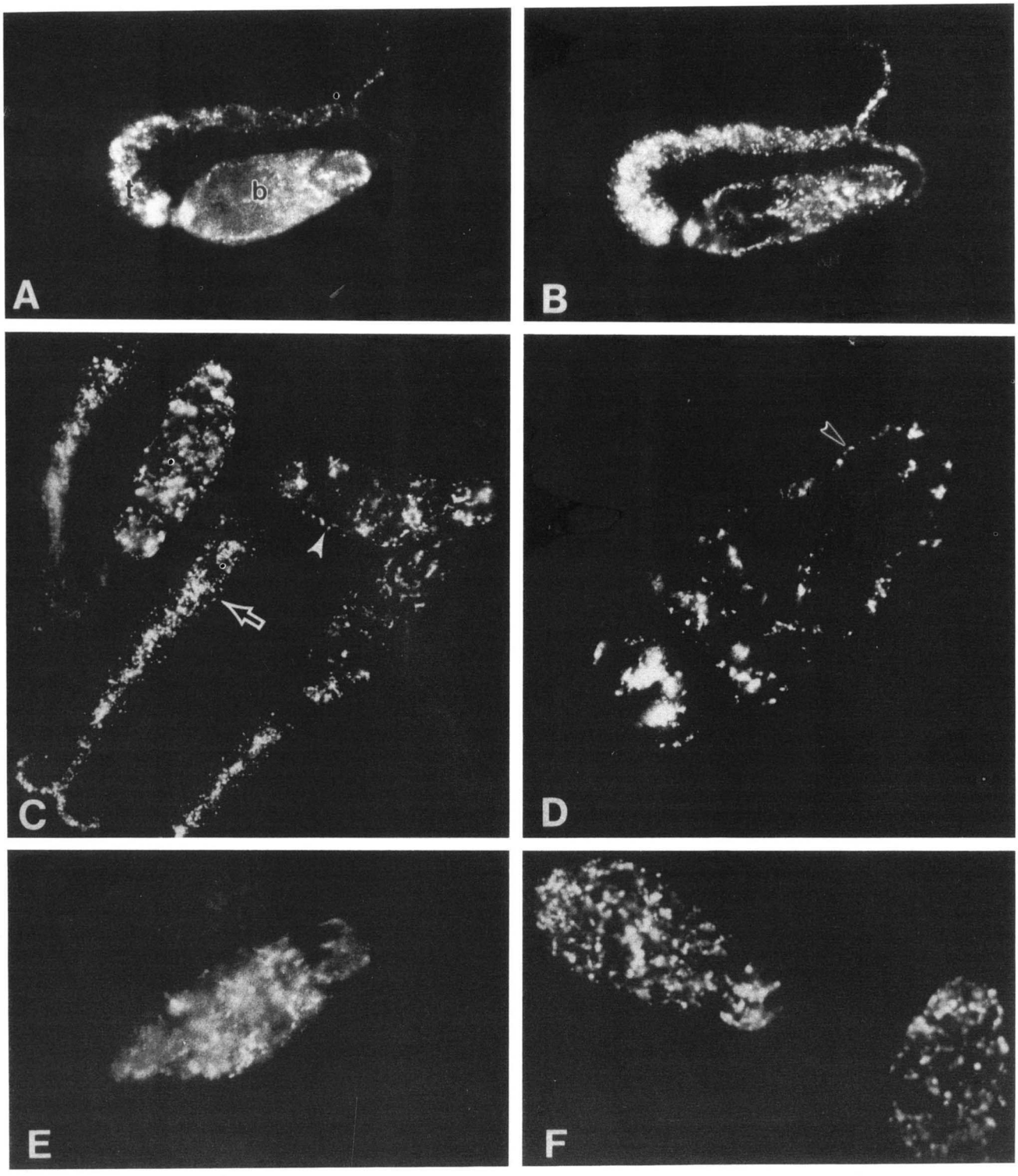

Fig. 1. Epifluorescent microscopic examination of the incorporation of Bodipy-C_{12} into cercariae and schistosomula in vitro. A) Cercaria incubated with 200 nM Bodipy-C_{12} for 1 h and viewed on the FITC cube; body (b), tail (t) (450×). B) The same cercaria as in A) except viewed on the rhodamine cube (450×). C) Cercaria labeled 1 h with 200 nM Bodipy-C_{12}, then washed, mechanically transformed, and viewed 3 h post-transformation. The preacetabular glands are not labeled in this newly transformed schistosomulum (arrowhead), however in another organism, the preacetabular glands are labeled. The tail (t) has a punctate distribution of lipid droplets (450×). D) Cercaria labeled 1 h with 2 μM Bodipy-C_{12}, then washed, mechanically transformed, and viewed 24 h post-transformation. Bodipy-C_{12} lipid was localized to the subtegumental region (arrowhead) (900×). E) Schistosomula labeled 1 h with 200 nM Bodipy-C_{12} and viewed immediately on the FITC cube (675×). F) Schistosomula labeled 1 h with 2 μM Bodipy-C_{12}, washed, and viewed 24 h later on the FITC cube (585×).

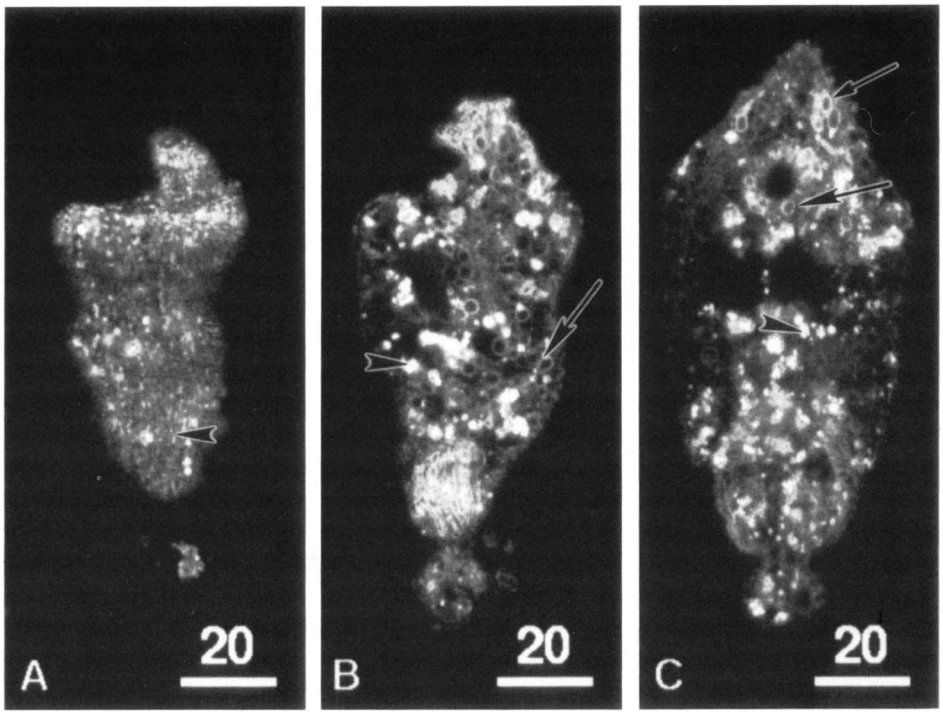

Fig. 2. Fluorescent confocal microscopy of Bodipy-C_{12}-labeled schistosomula. Parasites were labeled, incubated overnight, immobilized with 10^{-5} M eserine, placed in microwell chambers to prevent crushing, and then optically sectioned on the confocal microscope at 1-μm intervals. Three representative serial sections are shown. Optical sections recorded at 2 μm (A), 5 μm (B), and 15 μm from the surface of the organism reveal the distribution of Bodipy-C_{12} within the organism. (A) Optical section just below the organism surface reveals fluorescent labeling of longitudinal muscle fibers (arrowhead). (B) Lipid droplets (arrowheads) appear as bright punctate spots of variable size below the tegument of the organism. Cytoplasmic uptake of Bodipy-C_{12} is present in cytons (arrows). Note the exclusion of fluorescent label in the nuclei. (C) Lipid droplets (arrowhead) are present in the midgut. Acetabular cells have taken up the fluorescent label (large arrow). Cytons (small arrow) appear brightly labeled at the subtegumental margin of the organism.

Biochemical analysis

Biochemical analysis demonstrated a concentration-dependent increase in total Bodipy-C_{12} incorporation by schistosomula (**Fig. 3A**). A similar concentration-dependent increase was observed for incorporation of Bodipy-C_{12} into each of the major lipid classes (Fig. 3B). However, the relative incorporation into the major lipid classes was concentration-independent. For organisms labeled for 1 h approximately 31% of the label was incorporated into diglycerides, 2% into monoglycerides, and 5% into phospholipids, while greater than 60% remained as the free fatty acid analog. By contrast, after a 1-h incubation with the analog followed by overnight incubation only 10% remained as the free analog. The majority of the lipid residing in diglycerides, monoglycerides, and phospholipids (65%, 8%, and 10%, respectively).

To characterize the labeled phospholipid classes, lipids extracted from labeled organisms were analyzed by HPLC. In agreement with the results obtained by solid phase extraction, the majority of the Bodipy-C_{12} label was found in neutral lipids that eluted in the solvent front of a phospholipid HPLC separation (**Fig. 4**). However, labeling of worm phospholipids was also detected including PS, PC, and PE (Fig. 4).

In vivo labeling of cercariae in snails

In vitro labeling of either cercariae or schistosomula with Bodipy-C_{12} resulted in negligible surface labeling of the parasite, possibly because surface membranes originate from preformed lipid stores synthesized early in cercarial development. In an attempt to label such lipid pools at early developmental stages and to allow comparison of in vitro versus in vivo labeling of organisms, cercariae were labeled with Bodipy-C_{12} prior to release from the snail host. Yellow-gold fluorescence of cercariae released from snails incubated in Bodipy-C_{12} (**Fig. 5A**) was similar to cercariae labeled in vitro (Fig. 1A), but was less intense. Like in vitro labeled cercariae, labeling was evident throughout the cercariae but was most prominent in the anterior of the worm, in the ducts, and in the tail. Labeling in the ducts and in the glands was somewhat diffuse. In contrast, no fluorescence was detected in cercariae

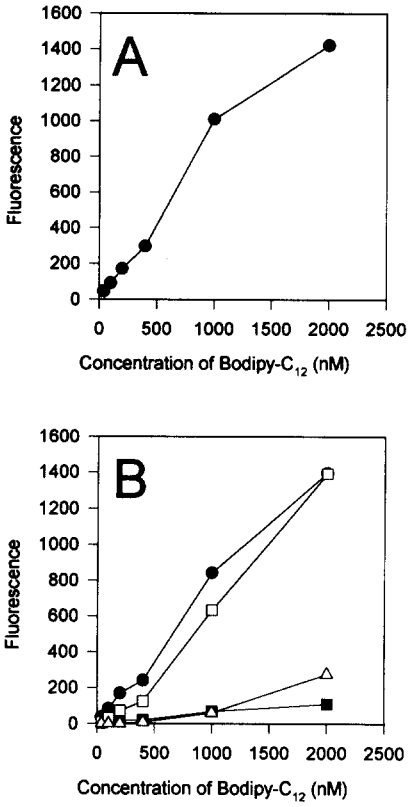

Fig. 3. Incorporation of Bodipy-C_{12} into parasite lipids. Schistosomula were labeled for 1 h with the concentration of Bodipy-C_{12} indicated and extracted. Fluorescence was measured in total lipid extract (A) or in lipid classes after separation by solid phase extraction (B). Lipid classes in panel B are fatty acids (closed circle), diglycerides (open square), monoglycerides (triangle), and phospholipids (closed square). Negligible fluorescence was found in the cholesteryl ester, cholesterol, and triglyceride fractions. Data shown is representative of three separate experiments.

released from control snails (not shown). To determine the persistence and toxicity of the Bodipy-C_{12} label, cercariae were shed from labeled snails at intervals from 4 days (Fig. 5B) to 21 days after labeling. A decrease in the intensity of labeling was evident with time but some labeling persisted up to 21 days after the initial labeling period. There was no difference in the localization of the fatty acid analog for cercariae shed at 0 days versus 21 days. Fatty acid analog was also evident in immature cercariae isolated from the hepatopancreas (not shown) and the labeling pattern was similar to that of the labeled free-swimming cercariae. Thus, these results show that Bodipy-C_{12} at the concentration used in these experiments is well tolerated by both the snail and the schistosome and is very persistent.

Three hours after transformation of labeled cercariae released from snails (Fig. 5C), the distribution of the fatty acid was distinctly different from that observed in cercariae but similar to the distribution observed in newly transformed schistosomula labeled in vitro (Fig. 1D). Like schistosomula labeled in vitro, yellow-gold fluorescence was localized to droplets dispersed throughout the schistosomula. No differences in the distribution of label were observed between schistosomula 3 h after transformation compared to those 24 h post-transformation (not shown). Unlike the parasites labeled in vitro, no red fluorescence was observed in either in vivo labeled cercariae or their derivative schistosomula indicating lower concentrations of label in these organisms than those labeled in vitro.

Labeling of schistosomula with fluorescent phospholipid analogs

In a previous study (8) we have shown that acyl-chain-labeled fluorescent phospholipid analogs localize to a subtegumental cellular network in schistosomula while a head group-labeled phospholipid stained only the parasite surface and gut. To extend these observations and to compare the distribution of labeled fatty acids to that of labeled phospholipids, parasites were labeled with three fluorescent phosphatidylcholine analogs, one labeled with NBD and the other two with Bodipy-C_{12}. These experiments demonstrated that C_5-bodipy-PC (**Fig. 6A**) and C_6-NBD-PC (Fig. 6C) were incorporated by the parasite into sub-tegumental structures exactly as described previously (8). By comparison, C_{12}-bodipy-PC was found primarily on the surface of the organism and in the acetabulum, with a slight amount in the gut (Fig. 6B). These results indicate that the longer chain length phospholipid analog was not incorporated as rapidly by the organism thus demonstrating specificity in the worms' transport processes based on phospholipid acyl chain content.

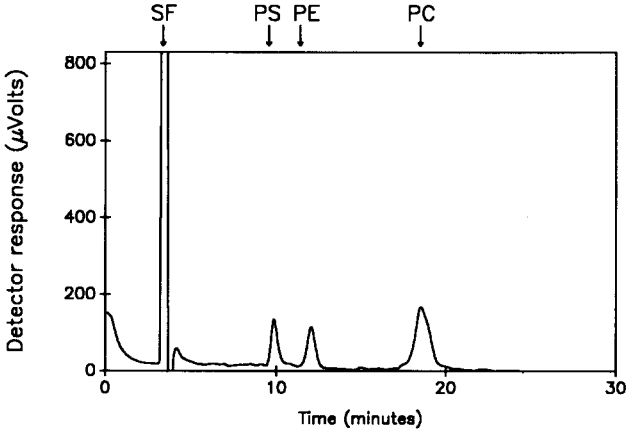

Fig. 4. HPLC analysis of Bodipy-C_{12} labeled phospholipids from schistosomula. Schistosomula were incubated 1 h with 200 nM Bodipy-C_{12}. Labeled organisms were washed, extracted, and separated by HPLC with fluorescence detection. The fatty acid analog was incorporated into both neutral lipids and phospholipids by the parasite. SF, solvent front; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

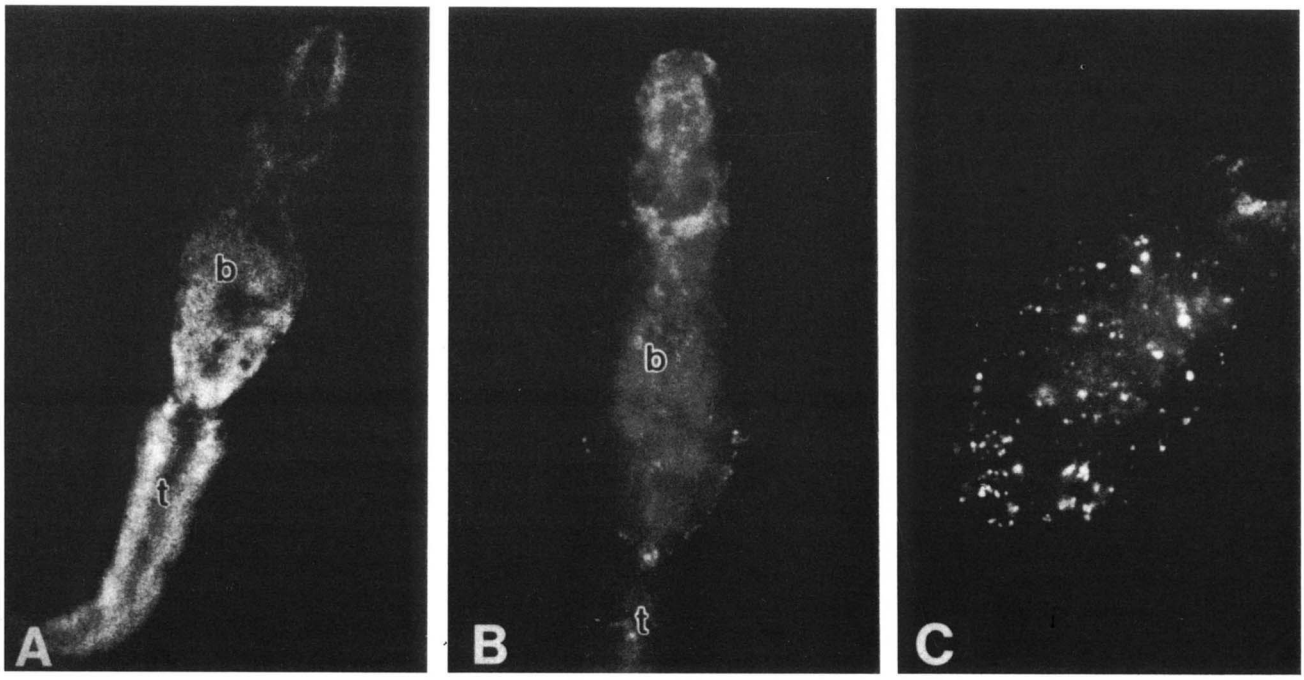

Fig. 5. Fluorescence associated with cercariae and schistosomula released from snails incubated with Bodipy-C_{12}. Snails were labeled 24 h at a final Bodipy-C_{12} concentration of 1.0 μM and washed, and cercariae were shed from labeled snails. Using epifluorescent microscopy, the distribution of label in bodies (b) and tails (t) released from labeled snails was similar to that observed when cercariae were labeled in vitro (675×) (A). The distribution of label from cercariae released after 4 days (900×) (B) did not differ from cercariae released after 21 days (data not shown). Distribution of the label in schistosomula prepared from cercariae released from labeled snails (C) was distinctly different from the corresponding cercaria but similar to schistosomula labeled in vitro (720×).

To provide spatial information on the distribution of the probes, serial section confocal microscopy was performed on samples incubated with C_6-NBD-PC. Results from these preparations confirmed that the majority of the acyl-chain-labeled phospholipids were localized to cytons within the parasite (Fig. 6C). The majority of the label was found in a subtegumental region around the worm 0.5–4.0 μm below the surface of the parasite. A partial three-dimensional reconstruction of an optically sectioned schistosomulum revealed the interconnecting nature of the labeled cells (Fig. 6D) and these results are consistent with the observation of a subtegumental syncytium. Little fluorescence was observed on the surface or in the interior of the worm. No labeling of the gut was evident. Therefore, the results obtained by confocal microscopy indicate that fluorescent NBD becomes localized to specific areas of the parasite.

To confirm and improve the resolution of the confocal microscopy results, a DAB photoconversion procedure was performed following confocal analysis. Photobleaching of worms containing NBD-PC with 488 nm laser light in the presence of DAB resulted in the deposition of a light brown precipitate which was made electron dense by treatment with 1% osmium tetroxide. Transmission electron microscopy revealed the deposition of the DAB–osmium tetroxide reaction product within well-defined cytoplasmic processes 2 to 5 μm below the tegumental surface of the worms (**Fig. 7**). The DAB Reaction product was absent in cell processes from control worms not labeled with C_6-NBD-PC.

DISCUSSION

Unlike the unicellular bacterial or protozoal pathogens, helminth parasites are multicellular organisms. Understanding lipid synthesis and processing by these organisms, therefore, requires both knowledge of the organism's biochemical processes and an understanding of cellular structures through which these processes are accomplished. In the present work, we have characterized the compartmentalization of fluorescent lipid analogs in two different stages of the parasite. These studies demonstrate that fluorescent fatty acid and phospholipid analogs are incorporated into cercariae and schistosomula of *S. mansoni* and that fluorescent fatty acid analogs can be metabolized into neutral lipids and phospholipids by this organism. Localization of the fluorescent analogs was dependent on the analog used, the parasite stage examined, and the length of time after incubation with the analog. Specifically, the fatty acid analog was diffusely distributed initially but localized to lipid droplets dispersed through-

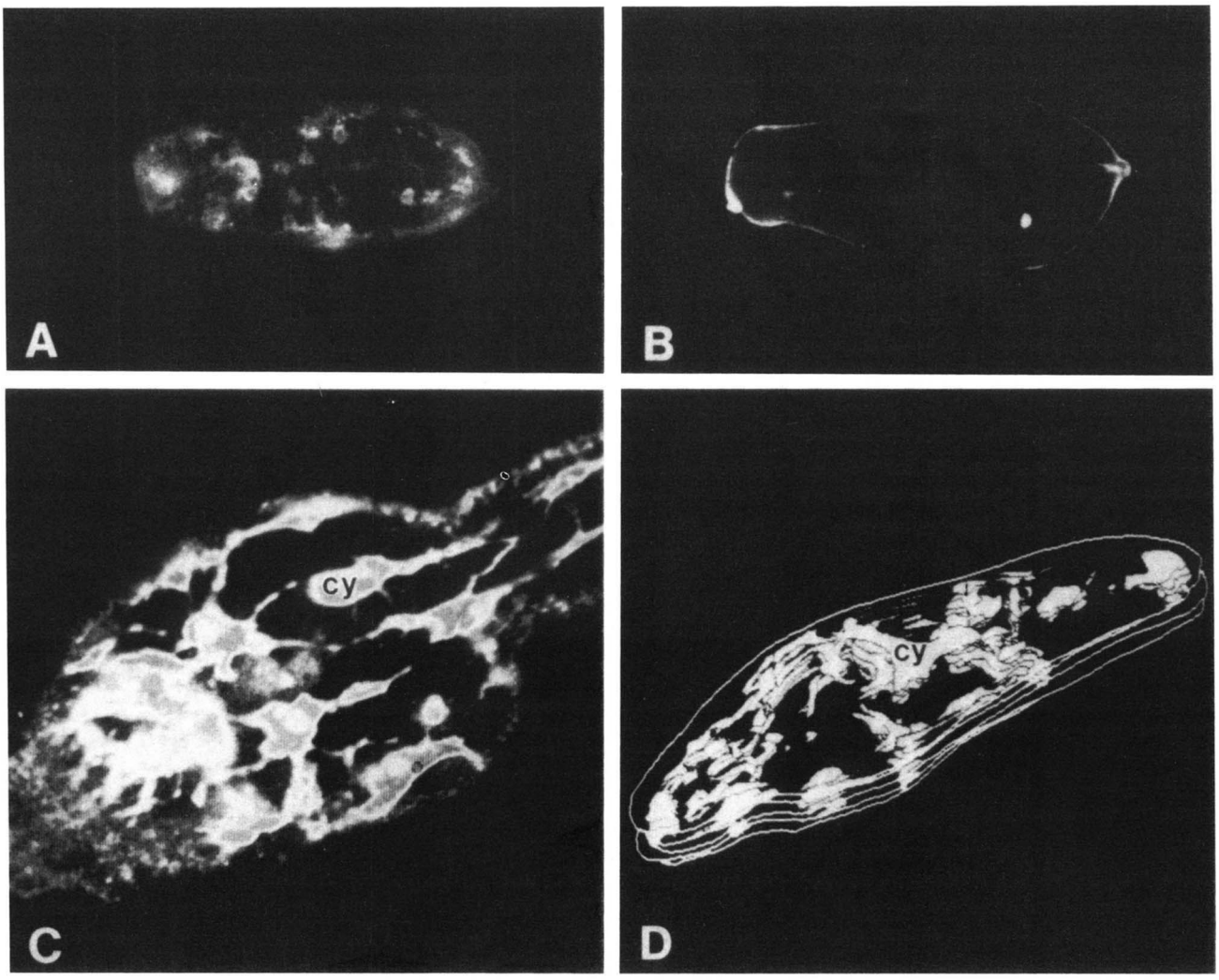

Fig. 6. Labeling of schistosomula with fluorescent phospholipid analogs. Schistosomula were labeled with liposomes containing either C_5-bodipy-PC (A), C_{12}-bodipy-PC (B), or C_6-NBD-PC (C). Figs. 6 A, B are epifluorescence micrographs, and Fig. 6C was taken by a Bio-Rad confocal microscope. Fig. 6D is a three-dimensional reconstruction made from serial optical sections, one of which is seen in Fig. C. C_5-bodipy-PC was incorporated into sub-tegumental regions of schistosomes (621×) (A), whereas, C_{12}-bodipy-PC was incorporated to the surface and gut regions (698×) (B). Intense cyton labeling was present in optical sections from a serially sectioned schistosome labeled with C_6-NBD-PC. The section shown was recorded 4 μm from the worm surface (1,530×) (C). A computer-assisted 3-D reconstruction (D) made from 25 serial optical sections represents the interconnected network of cytoplasmic processes 0.4 to 4.0 μm below the schistosome tegument. The worm surface is to the back of the reconstruction.

out both cercariae and schistosomula cultured further. For both stages, labeling of the parasite surface by fatty acid analogs was absent. By comparison, fluorescent phospholipid analogs labeled either the parasite surface and the gut, or specific sub-tegumental cellular structures. The phospholipid analogs, C_5-Bodipy-PC or C_6-NBD-PC, labeled cytons. However, C_{12}-Bodipy-PC labeled the surface of the parasite, the gut and occasionally, the acetabulum, but no sub-tegumental labeling was seen.

Little is known about the mechanism of lipid uptake in schistosomes. A protein has been identified in *S. mansoni* that has a high degree of sequence homology with fatty acid binding proteins from humans (19), but the functional significance of this protein is not clear. We have tried blocking uptake of radiolabeled fatty acids into schistosomula using antibody to fatty acid binding protein but were unsuccessful (unpublished data). Furthermore, several studies have shown that schistosomes incorporate fatty acids into phospholipids, neutral lipids, and proteins (5, 6, 20–22). However, none of these studies has yet demonstrated whether lipid synthetic capability is uniformly distributed throughout the worm. Furthermore, it is not yet clear whether fatty acids can enter the parasite by crossing the tegumental membranes or whether they must be absorbed through the gut. The biochemical results obtained in the present study are qualitatively similar to those described in mammalian cells but differ quantitatively. For example, both pyrene-

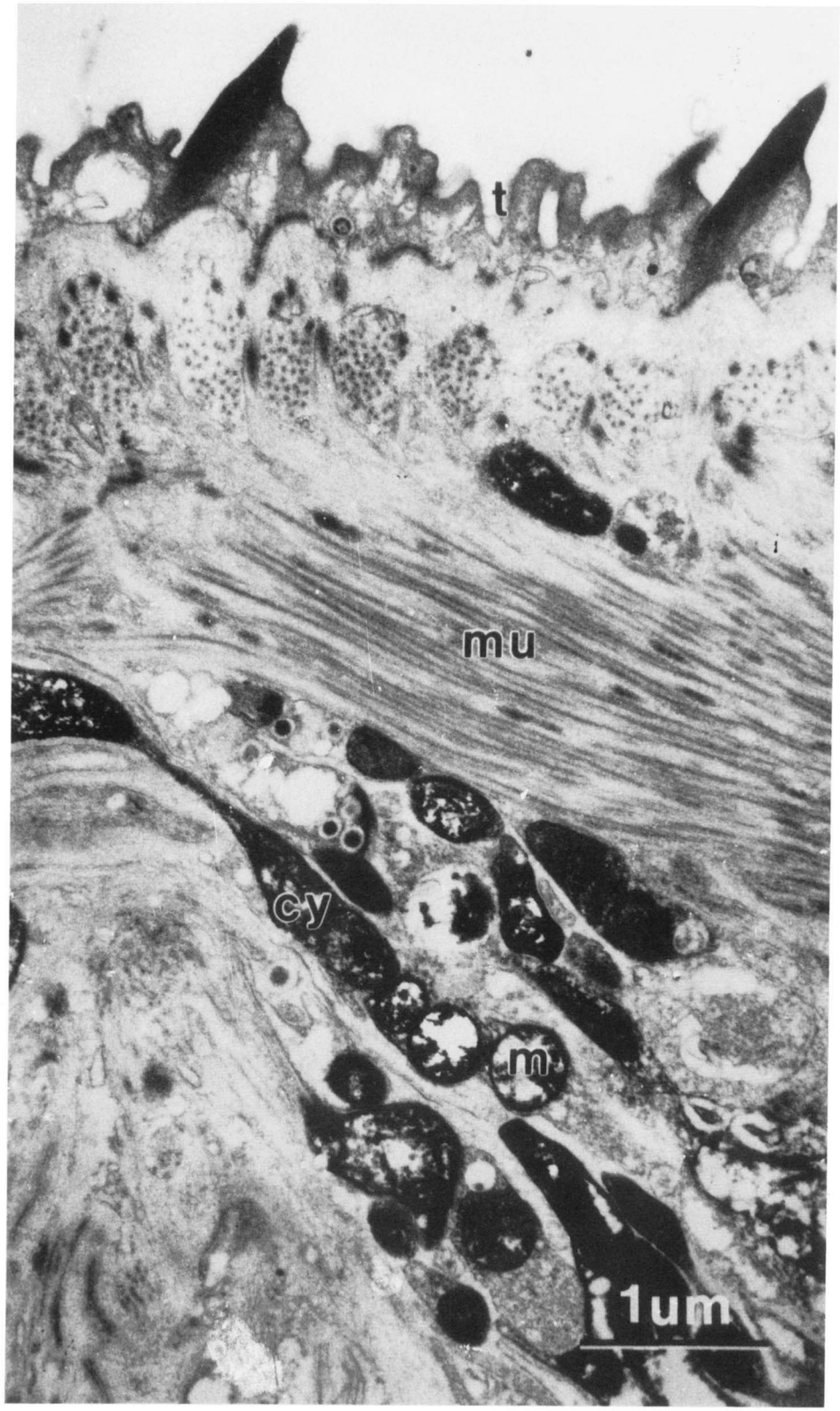

Fig. 7. Transmission electron micrograph showing the tegumental region of a C_6-NBD-PC labeled schistosome following photobleaching by laser light in the presence of DAB. Electron dense DAB reaction product was found within long cytoplasmic processes of cytons (cy) 2–5 μm from the tegumental surface (t) of the parasite, and below the muscular layer (mu). Mitochondria (m) in cytons were also labeled. Bar = 1 μm.

labeled and bodipy-labeled fatty acid analogs were incorporated by BHK cells into both neutral lipids, including diglycerides, and phospholipids, including phosphatidylcholine (23, 24). However, at either 2 or 8 h after incubation, triglycerides were the most heavily labeled neutral lipids (compared to diglycerides as shown here). Furthermore, phosphatidylcholine was labeled almost exclusively by the bodipy analog in BHK cells (23) compared to the results here showing significant labeling of phosphatidylserine and phosphatidylethanolamine in addition to phosphatidylcholine by schistosomes. As might be expected, then, the parasite appears to metabolize the analogs somewhat differently than mammalian cells.

There are at least three potential sources of fatty acids available to schistosomes, namely free fatty acids bound to serum proteins, or fatty acids derived from cellular triglycerides and phospholipids in host cell membrane and/or lipoproteins. Previous studies have shown host immune cell lysis upon contact or fuse to the parasite surface (5, 6). Ingestion of host cells has also been demonstrated (25). Lipoprotein binding to (26–28) and ingestion (29) by the parasite have also been demonstrated. Because of the parasite's lipid requirements, mechanism(s) of lipid acquisition represent a potentially important vaccine candidate or drug target.

The simplest interpretation of the results here is that the fatty acid and short chain phospholipid analogs are transported across the parasites' surface membranes and localized in a way that reflects the parasites' utilization of the native lipids. Localization of the fatty acids analogs into lipid droplets reflects the incorporation of fatty acids into complex neutral lipids by the organism in cells with lipid synthetic capability. Localization of the short chain phospholipids into cytons reflects that these cells are particularly rich in phospholipids. Furthermore, cytons may be important in phospholipid biosynthesis or transport, particularly of lipid components for the surface membranes. Although, our results suggest that some fluorescent fatty acid and phospholipid analogs are readily transported across the tegumental membrane, the parasite can also acquire lipids by ingestion and absorption through the gut. However, there are several reasons why it is unlikely that this was the sole or primary route of transport for these analogs. *1)* In the studies here and in our previous studies, two fatty acid analogs and four phospholipid analogs rapidly accumulated in the parasite with little or no labeling of the gut. *2)* Pulse-chase experiments showed no accumulation in the gut under any circumstances. *3)* Schistosomula do not have fully differentiated guts (25) and less transport by this route would be expected. Thus the most likely route for uptake of both fatty acids and those phospholipids found in internal structures of the worm was by transport across the tegumental membranes. It is possible that these analogs are absorbed so rapidly through the gut that no labeling was detectable; however, this seems unlikely. Furthermore, other studies have provided evidence that another labeled lipid, NBD-ceramide, is transferred across the tegumental surface of adult *S. mansoni* (30).

It is not clear why no appreciable fluorescence was found on the parasite surface after labeling with either fatty acid analogs. The lipids used for biogenesis of the tegumental membranes may be synthesized early in the development of cercaria and stored for utilization during cercarial transformation. Lipids incorporated by mature cercariae or schistosomula would, therefore, not be immediately utilized for tegumental membrane biogenesis. Alternatively, the lipid content of the surface membranes may represent such a small proportion of the total lipids in the organism that the apparent surface fluorescence is small, i.e., while there is actually a small amount of label which is incorporated into the surface membranes, it was flooded out by the label in the droplets. However, quantitative image analysis of digital confocal images do not detect differences between background and Bodipy-C_{12} label in these areas. Finally, although these probes are incorporated, as would be expected for native lipids, it is possible they are not incorporated into the surface membranes because there are specific phospholipid molecular species.

In previous studies, we showed by epifluorescence microscopy that when schistosomula were labeled with acyl chain-labeled NBD-PC and NBD-PE, these phospholipids were localized to cytons in the parasite. By comparison, head group-labeled fluorescein-PE was found only on the parasite surface, in the gut, and in the acetabulum. The results from the present study confirm and expand on these results. Like the NBD-labeled PC, C^6-Bodipy-labeled PC was localized in cytons, but C_{12}-bodipy-labeled PC was found only on the surface and in the gut. Thus, this is the first demonstration of differential transport into this parasite based on acyl chain length. Combining the results from these two studies we can now say that, as might be expected, phospholipid transport into the parasite is influenced both by acyl chain composition as well as head group structure.

There have been no previous studies concerned with Bodipy-C_{12} uptake by microorganisms, thus several points should be made concerning the suitability of these fluorescent fatty acid analogs for these studies. When schistosomula were incubated in high concentrations of Bodipy-C_{12}, viability was excellent even for extended culture. There was no difference in viability between organisms that were labeled with Bodipy-C_{12} versus those that were not. As has been previously observed, we found that under some Bodipy-C_{12}-labeling conditions fluorescence was observed on both FITC and rhodamine channels. The fluorescence on the rhodamine channel is presumably due to eximer emission and reflects a high concentration of analog in the parasite.

In this study, we have labeled cercariae and schistosomula in vitro and have shown labeling of cercariae of *Schistosoma mansoni* with a fluorescent fatty acid analog within the intermediate snail host, *Biomphalaria glabrata*. This labeling procedure is simple, rapid, persistent, and is nontoxic to both the parasite and its intermediate snail host. Thus this method may have some practical uses for studying lipid reorganization during transformation or as a potential tracking method for following parasites in vivo. In previous tracking studies, cercariae have been labeled with radioactive isotopes that are expensive and require special handling procedures (31, 32). The present method may simplify such studies.

The authors thank Bruce Ekstein for expert photographic assistance, Dr. Fred Lewis for providing infected snails, and Becky Schweighardt for critical reading of the manuscript. Supported by NIH grants AI24570 and NHLBI1F32.

Manuscript received 5 July 1993 and in revised form 24 June 1994.

REFERENCES

1. Nelson, G. S. 1989. The Role of Vaccines for the Control of Human Parasitic Diseases. CRC Press. 257–269.
2. Mitchell, G. F., M. V. Rogers, and W. U. Tiu. 1989. Schistosomiasis. CRC Press. 149–164.
3. Meyer, F., H. Meyer, and E. Bueding. 1970. Lipid metabolism in the parasitic and free-living flatworms. *Schistosoma mansoni* and *Dugesia dorotocephala*. *Biochim. Biophys. Acta.* **210:** 257–266.
4. Smith, T. N., T. J. Brooks, and V. G. Lockard. 1970. In vitro studies on cholesterol metabolism in the blood fluke *Schistosoma mansoni*. *Lipids.* **5:** 854–856.
5. Furlong, S. T., and J. P. Caulfield. 1989. *Schistosoma mansoni*: synthesis and release of phospholipids, lysophospholipids, and neutral lipids by schistosomula. *Exp. Parasitol.* **69:** 65–77.
6. Golan, D. E., C. S. Brown, C. M. Cianci, S. T. Furlong, and J. P. Caulfield. 1986. Schistosomula of *Schistosoma mansoni* use lysophosphatidylcholine to lyse adherent human red blood cells and immobilize red cell membrane components. *J. Cell Biol.* **103:** 819–828.
7. Furlong, S. T. 1991. Unique roles for lipids in *Schistosoma mansoni*. *Parasitol. Today.* **7:** 59–62.
8. Furlong, S. T., K. S. Thibault, and R. A. Rogers. 1992. Fluorescent phospholipids preferentially accumulate in subtegumental cells of schistosomula of *Schistosoma mansoni*. *J. Cell. Sci.* **108:** 823–830.
9. Ramalho-Pinto, F. J., G. Gazzinelli, R. E. Howells, T. A. Mota-Santos, E. A. Figueiredo, and J. Pelligrino. 1974. *Schistosoma mansoni*: defined medium for stepwise transformation of cercaria to schistosomule in vitro. *Exp. Parasitol.* **36:** 360–370.
10. Samuelson, J. C., J. P. Caulfield, and J. R. David. 1980. *Schistosoma mansoni*: post-transformational surface changes in schistosomula grown in vitro and in mice. *Exp. Parasitol.* **50:** 369–383.
11. Samuelson, J. C., J. J. Quinn, and J. P. Caulfield. 1984. Video microscopy of swimming and secreting cercariae of *Schistosoma mansoni*. *J. Parasitol.* **70:** 996–999.
12. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
13. Kaluzny, M. A., L. A. Duncan, M. V. Merritt, and D. E. Epps. 1985. Rapid separation of lipid classes in high yield and purity using bonded phase columns. *J. Lipid Res.* **26:** 135–140.
14. Furlong, S. T., and J. P. Caulfield. 1988. *Schistosoma mansoni*: sterol and phospholipid composition of cercariae, schistosomula and adults. *Exp. Parasitol.* **65:** 222–231.
15. Chen, S. S., and A. Y. Kou. 1982. Improved procedure for the separation of phospholipids by high-performance liquid chromatography. *J. Chromatogr.* **227:** 25–31.
16. Young, S. J., S. M. Royer, P. M. Groves, and J. C. Kinnamon. 1987. Three-dimensional reconstructions from serial micrographs using the IBM PC. *J. Electron Microsoc. Tech.* **6:** 207–217.
17. Pagano, R. E., M. A. Sepanski, and O. C. Martin. 1989. Molecular trapping of a fluorescent ceramide analog at the Golgi apparatus of fixed cells: interactions with endogenous lipids provides a trans Golgi marker for both light and electron microscopy. *J. Cell. Biol.* **109:** 2067–2079.
18. Pagano, R. E., O. C. Martin, H. C. Kang, and R. P. Haugland. 1991. A novel fluorescent ceramide analog for studying membrane traffic in animal cells: accumulation at the Golgi apparatus results in altered spectral properties of the sphingolipid precursor. *J. Cell Biol.* **113:** 1267–1279.
19. Moser, D., M. Tendler, G. Griffiths, and M. Klinkert. 1991. A 14-kDa *Schistosoma mansoni* polypeptide is homologous to a gene family of fatty acid binding proteins. *J. Biol. Chem.* **266:** 8447–8454.
20. Furlong, S. T., and J. P. Caulfield. 1991. Head group precursors modify phospholipid synthesis in *Schistosoma mansoni*. *J. Lipid. Res.* **32:** 703–712.
21. Rumjanek, F. D., and A. J. Simpson. 1980. The incorporation and utilization of radiolabelled lipids by adult *Schistosoma mansoni* in vitro. *Mol. Biochem. Parasitol.* **1:** 31–44.
22. Wiest, P. M., E. J. Tisdale, W. L. Roberts, T. L. Rosenberry, A. A. Mahmoud, and A. M. Tartakoff. 1988. Characterization of [^{3}H]palmitate and [^{3}H]ethanolamine-labeled proteins in the multicellular parasitic trematode *Schistosoma mansoni*. *Biochem. J.* **254:** 419–426.
23. Kasurinen, J. 1992. A novel fluorescent fatty acid, 5-methyl-bdy-3dodecanoic acid, is a potential probe in lipid transport studies by incorporating selectively to lipid classes of BHK cells. *Biochem. Biophys. Res. Commun.* **187:** 1594–1601.
24. Kasurinen, J., and P. Somerharju. 1992. Metabolism of pyrenyl fatty acids in baby hamster kidney fibroblasts: effect of the acyl chain length. *J. Biol. Chem.* **267:** 6563–6569.
25. Bogitsch, B. J. 1989. Observations on digestion in schistosomes or "blood and guts." *Trans. Am. Microsc. Soc.* **108:** 1–5.
26. Rumjanek, F. D., E. G. Campos, and L. C. C. Afonso. 1988. Evidence for the occurrence of LDL receptors in extracts of schistosomula of *Schistosoma mansoni*. *Mol. Biochem. Parasitol.* **28:** 145–152.
27. Rogers, M. V., K. J. Henkle, N. H. Fidge, and G. F. Mitchell. 1989. Identification of a multispecific lipoprotein receptor in adult *Schistosoma japonicum* by ligand blotting analysis. *Mol. Biochem. Parasitol.* **35:** 79–88.
28. Chiang, C-P., and J. P. Caulfield. 1989. Human lipoprotein binding to schistosomula of *Schistosoma mansoni*: displacement by polyanions, parasite antigen masking, and persistence in young larvae. *Am. J. Pathol.* **135:** 1015–1024.
29. Bennett, M. W., and J. P. Caulfield. 1991. Specific binding of human low-density lipoprotein to the surface of the schistosoma of *Schistosoma mansoni* and ingestion by the parasite. *Am. J. Pathol.* **138:** 1173–1182.
30. Moffat, D., and J. R. Kusel. 1992. Fluorescent lipid uptake and transport in adult *Schistosoma mansoni*. *Parasitology.* **105:** 81–89.
31. Dean, D. A., and B. L. Mangold. 1984. Autographic analysis of resistance to reinfection with *Schistosoma mansoni* in mice. *Am. J. Trop. Med. Hyg.* **33:** 97–103.
32. Dean, D. A., B. L. Mangold, J. R. Georgi, and R. H. Jacobson. 1984. Comparison of *Schistosoma mansoni* migration patterns in normal and irradiated cercaria-immunized mice by means of autographic analysis. *Am. J. Trop. Med. Hyg.* **33:** 89–96.