Loss of Covalently Labeled Glycoproteins and Glycolipids from the Surface of Newly Transformed Schistosomula of *Schistosoma mansoni*

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**ABSTRACT** Schistosomula of *Schistosoma mansoni* were labeled by oxidation with galactose oxidase or with periodate followed by reduction with NaB₃H₄ to study the loss of the surface membrane of these parasites in vitro. Grain counts of light microscope autoradiographs (LMARG) of radiolabeled schistosomula show that both galactose oxidase and periodate specifically label the surface of the organisms. Galactose oxidase labels 11 glycoproteins on the surface of skin and mechanical schistosomula, ranging in apparent molecular weight from 17,000 to >105,000. These glycoproteins are lost from the surface of schistosomula with a halftime of 10-15 h in culture in defined medium. Most of these glycoproteins appear to be shed intact from the surface of the schistosomula rather than endocytosed and degraded, because >50% of each of the lost proteins can be recovered by trichloroacetic acid precipitation of the culture medium and because there is no internalization of the radiolabels into cultured schistosomula examined by LMARG. In addition to glycoproteins, periodate labels at least seven glycolipids on the surface of mechanical schistosomula. After culture for 15 h, more than half of each of these periodate-labeled proteins and lipids are lost from the schistosomula, and their abundance relative to each other remains similar to that of freshly labeled organisms. Since both proteins and lipids are lost from the surface of the schistosomula at the same rate, we believe that we are observing a general loss of the parasite surface membrane.

**MATERIALS AND METHODS**

**Experimental Design**

The strategy of these experiments was to surface-label schistosomula with galactose oxidase or periodate oxidation followed by reduction with NaB₃H₄ and to study the turnover of these radiolabeled surface proteins by cultured schistosomula. The same experimental protocol for studying the clearance of ¹²⁵I-Con A from the surface of schistosomula (19) was repeated exactly: schistosomula were radio-labeled, cultured, and the amount and molecular weight of radiolabeled protein remaining on cultured schistosomula was examined by SDS PAGE. Surface labeling of the organisms and the rate of loss of radiolabeled proteins was examined by light microscope autoradiography (LMARG). In addition, lipids labeled by periodate and NaB₃H₄ were characterized by thin layer chromatography (TLC).

**Protocol for Surface-labeling Schistosomula with Galactose Oxidase or Periodate**

Schistosomular surface molecules were oxidized with galactose oxidase or periodate and reduced with NaB₃H₄ by established procedures (8, 9). Freshly prepared skin or mechanical schistosomula (19) were washed three times in
solubilized in SDS and reduced with mercaptoethanol. Glycoproteins in the schistosomula were incubated with NaB₃I-L. After reduction, organisms were washed six times with PBS. Radio-labeled schistosomula, 2,000-10,000/ml, were cultured in RPMI-BSA for 12-24 h at 37°C under 5% CO₂ and 95% O₂ (18).

To quantitate the distribution of radioactivity on the surface and on the interior of galactose oxidase- and periodate-labeled schistosomula and of control organisms treated with NaB₃H₄ alone, radioautographs were developed after 1-2 wk. Photographs of the northwest quadrant of the first section observed on each slide were taken at x 100 and enlarged to x 700. These photographs were overlaid with a translucent grid made up of 6 x 6 mm squares to determine the background radioactivity (grain density on the plastic section but not on the organisms) and of grains on the interior (used to calculate the absolute number of grains on the interior) was determined by counting the number of squares whose upper rim.

**Light Microscope Autoradiography of Surface-labeled Schistosomula**

Schistosomula at the end of the labeling procedure and after culture were washed six times in PBS and fixed in aldehydes, postfixed in OsO₄, and embedded in Epon as for transmission microscopy (2). Sections, 0.4 mm thick, with rose to green interference colors, were dried onto clean glass slides, coated with NTB-2 emulsion (Kodak), and exposed for 1-4 wk at 4°C before development in Kodak D-19 (17). Radioautographs, unstained or lightly stained with a dilute solution of Azure blue, were viewed with a Leitz Orthoplan microscope in bright field and photographed at a primary magnification of x 100-150 on Pan-X or Hi-Contrast Copy film (Kodak).

To quantify the distribution of radioactivity on the surface and on the interior of galactose oxidase- and periodate-labeled schistosomula and of control organisms treated with NaB₃H₄ alone, radioautographs were developed after 1-2 wk. Photographs of the northwest quadrant of the first section observed on each slide were taken at x 100 and enlarged to x 700. These photographs were overlaid with a translucent grid made up of 6 x 6 mm squares to determine the background radioactivity (grain density on the plastic section but not on the organisms) and of grains on the interior (used to calculate the absolute number of grains on the interior) was determined by counting the number of squares whose upper rim.

To measure the radioactivity on the surface of schistosomula, the translucent grid was removed from the photographs, and a line 1.5 mm thick was drawn along the edge of each organism. The number of grains falling on this line was counted, and the length of this line was measured with a map measure. The density of grains on the surface of each organism was then calculated by dividing the number of grains counted on the line by the length of the line x 1.5 mm, all minus the density of grains on the inside of the organism. The absolute number of grains on the surface was determined by subtracting the number of grains counted on the line the contributions of the inside and background.

Four values were used to statistically compare the distribution of radioactivity on the surface and on the interior of normal and peroxidase-labeled schistosomes: (a) surface grain density, (b) interior grain density, (c) ratio of grain density surface/inside, and (d) ratio of absolute number of grains surface/inside. Grain densities were corrected for enlargement and expressed in grains/μm². The average, standard deviation, and standard error of each of these four values for 12-20 organisms per experimental group were determined for galactose oxidase- and periodate-labeled schistosomula and statistically compared with values for organisms treated with NaB₃H₄ alone using a Student's t-test.

**TLC of Radio-labeled Lipids**

Radio-labeled lipids from 10,000 organisms per experimental condition were extracted from schistosomula immediately after labeling or after 15-h culture by sonication of organisms in 400 μl of methanol containing 20 μl of 0.1 N KCl and 0.01 M EDTA and by successive incubations for 30 min in 20 ml of chloroform:methanol in the ratios of 2:1, 1:1, and 1:2 (6).

1. Extracted lipids were filtered through sintered glass, dried on a rotovaporaator, and partitioned into aqueous and organic phases by resuspension in chloroform:methanol:water (C:M:H₂O) 8:4:3 (7). Lipids in the organic phase were chromatographed on 20-cm silica gel H plates (Analtech, Inc., Newark, DE) with C:M:H₂O 65:35:10 as the solvent, while aqueous phase lipids were separated on silica gel G plates with C:M:H₂O 55:45:10 as the solvent. Standard lipids run in parallel included monosialolganglioside, disialoganglioside, sphingomyelin, ceramide trihexoside, lactosyl ceramide, and ceramide (Supelco, Inc., Bellefonte, PA).

2. TLC plates were sprayed with primuline to detect standards and abundant schistosomulinar lipids (25), observed under ultraviolet light, and photographed. TLC were then scanned with ENHANCE for fluorography, exposed to preflashed film for 4-14 d at ~70°C, developed, and scanned as above.

**RESULTS**

**Localization of Specific and Nonspecific Radioactivity on Thick Sections of Schistosomula**

LMARG of schistosomula incubated with NaB₃H₄ alone shows silver grains diffusely distributed on the surface and inside of the parasites (Fig. 1). The grain densities on the surface and the interior are the same by statistical analysis (all P for three experiments >2), which indicates that there is no surface-labeling in the absence of an oxidant (Table 1). In contrast, schistosomula immediately after labeling with galactose oxidase or with periodate have silver grains on the surface of the organisms (Fig. 2). The density of the surface radioactivity on galactose oxidase- and periodate-labeled schistosomula is 17-22 and 15-20 times (ranges of three experiments), respectively, that on the surface of control organisms. This indicates significant (all P < 0.001) specific surface labeling by these two oxidants (Table 1). The density of grains on the inside of galactose oxidase- and periodate-labeled schistosomula, however, is statistically indistinguishable from that on the insides of control organisms (all P > 2), indicating no interior labeling of the organisms after oxidation procedures (Table 1).

The ratio of absolute grains on the surface (specific) and on the interior (nonspecific) of galactose oxidase- and periodate-
labeled schistosomula is 2.9–6.6:1 and 2.4–2.6:1, respectively (Table I). Schistosomula labeled with galactose oxidase and then cultured for 15 h show a 50–70% decrease in grain density on their surface, suggesting a 10–15-h halftime for the loss of specific label (Table I). There is a simultaneous 70–80% decrease in the nonspecific radioactivity in the interior of cultured organisms (Table I). The ratio of density of grains on the surface to those on the interior of cultured schistosomula increases to 52–54:1, while the ratio of the absolute number of grains on the surface to interior increases to 4.6–5.2:1 (Table I). This suggests that cultured schistosomula do not internalize the radioactivity into stable pools.

**Fluorography of Glycoproteins Labeled by Galactose Oxidase and Periodate on the Surface of Schistosomula**

Schistosomula incubated with NaB\(^3\)H\(_4\) alone do not incorporate \(^3\)H into protein (Fig. 3a). In contrast, 12 proteins ranging in apparent molecular weight from 17,000 to >105,000 are...
labeled when schistosomula are oxidised by galactose oxidase or periodate before reduction (Fig. 3). Skin schistosomula labeled with galactose oxidase show three major peaks of radioactivity at 17,000, 22,000, and 34,000 mol wt and minor peaks at 39,000, 41,000, 44,000, 54,000, 60,000, and 67,000. The two unmarked peaks at the left of peak A are two peaks representing unreacted borohydride running at the dye and tritiated lipids running ahead of the dye, much of which radioactivity is lost with time in culture. The stack is on the right.

Loss of Radio-labeled Glycoproteins from the Surface of Cultured Schistosomula

Cultured schistosomula lose surface glycoproteins labeled with either galactose oxidase or periodate. The loss of radio-labeled proteins is reflected in a decreased height of peaks of radioactivity in densitometric scans of fluorographs of mechanical schistosomula labeled and then cultured for 15 h (Fig. 3 b and c). The molecular weights and relative amounts of proteins \( \geq 17,000 \) on cultured schistosomula are similar to those found on freshly labeled schistosomula. In contrast, radioactivity below 17,000 running at or in front of the dye is lost more rapidly with time in culture.

The loss of surface-labeled glycoproteins was quantified by cutting and counting tube gels of freshly labeled schistosomula, cultured schistosomula, and TCA precipitates of the culture medium. Schistosomula labeled with galactose oxidase were chosen for quantification because more radioactivity over a wide molecular weight range was incorporated with galactose oxidase than with periodate (Fig. 3). The tube gels (Fig. 4) have a single 11% concentration of acrylamide and so have less fine resolution than the gradient slab gels (Fig. 3). At zero time, four major protein peaks of radioactivity, A–C and stack, are apparent on tube gels (Fig. 4). These peaks correspond to the peaks seen on slab gels as follows: (A) 17,000–19,000; (B) 22,000–27,000; (C) 39,000–60,000 and stack >80,000 mol wt (Fig. 4). The double peak <17,000 on gradient gels (Fig. 3) is seen on tube gels as a single peak (Fig. 4). The amount of radioactivity in the protein peaks in freshly labeled organisms...
averaged 28% of the total in A, 24% in B, 13% in C, and 10% in stack (Fig. 4 and Table II). After 12-h culture, the amount of radioactivity in each of these protein peaks is reduced to 40-49% of initial labeling (Fig. 4 and Table II), which indicates that most surface-labeled proteins are being lost with a halftime of 10-12 h. Some 25-34% of the zero-time labeled protein is recoverable in the culture medium by TCA precipitation (Fig. 4 and Table II). This radioactivity is equivalent to 50-60% of the radioactivity that is lost from the surface of organisms during culture. The TCA-precipitable proteins have a similar molecular weight distribution as the proteins on freshly labeled or cultured schistosomula. By calculation, 22-26% of the initial radioactivity cannot be recovered, distribution over all molecular weight ranges ≥17,000 (Table II). However, most of the radioactivity at the front, 25-30% of the initial labeling, is lost from cultured schistosomula and not recovered in the medium (Fig. 4 and Table II).

**Periodate-labeling of Schistosomula**

*Surface Lipids*

\[ \text{NaB}^3\text{H}_4 \] alone labels nonspecifically some schistosomular lipids which partition into both phases of a Folch extraction. TLC of these nonspecific labeled lipids show a number of discrete spots, most of which are lost when organisms are cultured for 15 h after labeling. The nonspecific labeling with \[ \text{NaB}^3\text{H}_4 \] can be largely removed by pretreating organisms with 30 μg/ml cold NaBH₄ for 30 min at room temperature before oxidation. Periodate specifically labels glycolipids which partition predominantly into the aqueous phase. On TLC these periodate-labeled lipids run in the neutral rather than acidic region (Fig. 5). Major labeled peaks chromatograph similar to ceramide trihexoside (Rₚ = 0.35) and lactosyl ceramide (Rₚ = 0.35). Five other major peaks have Rₚ values between 0.5 and 0.85. When schistosomula are cultured for 15 h after periodate labeling, >50% of each lipid peak is lost, but the Rₚ values and relative abundance of most of the lipids remain the same as those seen after the initial labeling (Fig. 5). Galactose oxidase did not reliably label any schistosomular glycolipids.

**Table II**

| Molecular weight range | Zero time | 12-h cult | TCA ppt | nonTCA ppt |
|------------------------|-----------|-----------|---------|------------|
| Front                  | 30+/−13   | 7.6+/−2   | 1.8+/−1 | 21+/−12    |
|                        | (100)     | (26+/−8)  | (12+/−7) | (70+/−10)  |
| Peak A                 | 17,000    | 14+/−4    | 8+/−1   | 8+/−7      |
|                        | (100)     | (48+/−5)  | (29+/−6) | (22+/−10)  |
| Peak B                 | 22,000–27,000 | 8+/−3    | 7.3+/−3 | 5+/−2     |
|                        | (100)     | (40+/−6)  | (34+/−3) | (26+/−2)   |
| Peak C                 | 39,000–67,000 | 5.3+/−3  | 4.1+/−1 | 4.5+/3    |
|                        | (100)     | (40+/−6)  | (33+/−10)| (26+/−6)   |
| Stack                  | 5.9+/−2   | 2.8+/−1   | 2.1+/−1 | 2.0+/−2    |
|                        | (100)     | (49+/−9)  | (25+/−8) | (24+/−10)  |

Summary of data from three experiments performed as in Fig. 4 in which schistosomula were labeled with galactose oxidase. The amount of radioactivity remaining with cultured organisms and recoverable by TCA precipitation from the culture medium was determined by counting and cutting SDS tube gels. The radioactivity lost from the schistosomula which is not recovered by acid precipitation (nonTCA ppt) was calculated by subtracting the sum of the radioactivity of the cultured organisms and the media precipitates from the radioactivity bound to schistosomula at zero time. Radioactivity is expressed as counts per minute (cpm) x 10⁻⁷/25,000 organisms and is the average and standard deviations for the three experiments. The percentage of zero-time radioactivity (arbitrarily defined as 100%) in the front, protein peaks A–C, and stack, which remains with schistosomula after culture or is precipitable (ppt) or nonprecipitable in the culture medium, is given in parentheses.
Schistosomula of *S. mansoni* were labeled by oxidation with galactose oxidase or periodate followed by reduction with NaBH₄ to study the fate of the surface membrane of the parasites in vitro. By light microscope autoradiography, both techniques specifically and exclusively label the surface of freshly transformed schistosomula, although some nonspecific radioactivity, quantitatively equivalent to that of organisms treated with NaBH₄ alone, is apparent within labeled organisms. Galactose oxidase labels 11 proteins, ranging in apparent molecular weight from 17,000 to >105,000, all of which are lost with a halftime of 10–15 h in culture in defined medium. Greater than 50% of these lost proteins are recoverable by TCA precipitation of the cultured medium, suggesting that much of the parasite surface is shed with proteins intact. Periodate labels glycoproteins similar to those labeled by galactose oxidase, and also labels at least seven glycolipids on the surface of the parasite. After culture for 15 h, >50% of each of these periodate-labeled proteins and lipids are lost from the schistosomula, parasite. After culture for 15 li, >50% of each of these periodate-labeled proteins and lipids are lost from the schistosomula, parasite. After culture for 15 li, >50% of each of these periodate-labeled proteins and lipids are lost from the surface of schistosomula. First, the surface of newly transformed schistosomula is complex, containing at least 12 different glycoproteins, which contain sialic acid and galactose, and seven different glycolipids. Second, similar sets of glycoproteins are labeled with galactose oxidase on the surface of skin and mechanical schistosomula. Because mechanical schistosomula were prepared in macromolecule-free medium, these proteins must be derived from the parasite and cannot be absorbed from host tissue or serum. Third, many of the glycoproteins labeled by galactose oxidase and periodate appear to have also been labeled on the surface of schistosomula by iodosulfanilic acid (apparent molecular weights: 27,000, 34,000, 41,000, 67,000, 80,000, and 105,000) (24), lactoperoxidase (22,000, 27,000, 34,000, 41,000, 44,000, 54,000, 67,000, 80,000, and 105,000) (4, 16, 21), or 3-azido-4-iodo-naphthalene 2,7-disulfonic acid (34,000, 41,000, and 60,000) (D. Pratt, personal communication), suggesting that most of the schistosomular surface proteins may be glycoproteins. Fourth, polar glycolipids on the surface of schistosomula likely contain sialic acid and not galactose, because they are labeled by periodate but not galactose oxidase.

The viability of the schistosomula is unaffected by the surface labels so that we were able to study the rate and route of clearance of radiolabeled glycoproteins and glycolipids from the surface of cultured schistosomula. In general, the rate of loss of galactose oxidase- and periodate-labeled proteins from schistosomula is independent of molecular weight, so that the relative amount of each protein remaining on cultured schistosomula is similar to that on freshly labeled organisms. In the same way, the pattern of periodate-labeled glycolipids on cultured schistosomula is similar, though not identical, to that found initially. Quantitative measurements indicate an average halftime of glycoprotein loss to be 10–12 h. This halftime is in agreement with radioautography which showed that 50–60% of the surface-labeled radioactivity is lost in 15 h. Cultured schistosomula appear to lose surface labeled glycoproteins by shedding them into the medium. We were able to recover 50–60% of each molecular weight region of the labeled proteins by TCA precipitation from the culture medium. 22–26% of the labeled proteins were not recovered, presumably because of absorption of the protein onto glass and plastic and incomplete solubilization after precipitation. Although it is possible that some of the proteins were lost by degradation, either on the surface of the organism or in the culture media, it appears that most of the surface-labeled proteins are shed intact. How the proteins are shed is unclear. If the proteins are not inserted into the lipid bilayer, they could simply fall off. However, all of the proteins labeled by iodosulfanilic acid, except the 68,000 g mol wt protein cannot be solubilized with high salt and require either SDS or Nonidet P-40 to remove them from the parasite, suggesting that they are tightly bound to the membrane (24). If, in fact, the proteins are inserted into the lipid bilayer, they could be shed either as membrane fragments or as micelles. We favor the interpretation that the proteins are shed with lipids because labeled glycolipids are lost from the parasite at about the same rate as the proteins. In addition, we believe that the proteins are shed as micelles because attempts to recover the radioactive proteins from the culture medium both by pelleting for 24 h at 100,000 g and by floatation into sucrose gradients, such as is done in subcellular fractionation (5), failed. This conclusion, that the membrane is shed in micelles, is also in agreement with the experiments described in our companion paper (3) in which neutrophils are shown to endocytose Con A together with galactose oxidase-borohydride labeled surface proteins from the parasite, yet the structure of the outer membrane remains unaltered by both thin-section and freeze-fracture.

The loss of labeled proteins and lipids from cultured schistosomula is similar to that observed with Con A, which was shed intact from cultured organisms with a halftime of 8–10 h (19). Since antischistosomal antibodies and complement are also lost at a similar rate (20), it appears that molecules bound to the surface of schistosomula are being lost at about the same rate as the parasite surface itself. The parasite, then, may defend itself in part by shedding antibody and complement (and the parasite molecules to which they are bound) as part of the normal turnover of its surface.

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