Differences in Expression and Exposure of Promastigote and Amastigote Membrane Molecules in *Leishmania tropica*

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The accessibility of particular *Leishmania tropica* promastigote (extracellular) and amastigote (intracellular) membrane molecules might be related to the relative abilities of the two stages to induce host immune responses. To examine the exposure of membrane antigens on resident macrophage-susceptible promastigotes and resident macrophage-resistant amastigotes, both stages were analyzed by polyacrylamide gel electrophoresis and immunoblotting after specific labeling and extraction procedures. Protein compositional studies, using metabolic labeling of promastigotes and amastigotes, demonstrated that both forms possessed numerous endogenously synthesized proteins. In addition, a marked difference was revealed in the external exposure of promastigote and amastigote membrane constituents when analyzed by ^{125}I surface labeling or Western blot analysis. Whereas nine promastigote proteins were intensely to moderately iodinated, only one amastigote membrane component was similarly labeled (9.5K band). Western blot analyses with serum from a rabbit immunized with a mixture of both *L. tropica* stages indicated that the majority of promastigote molecules accessible to ^{125}I may also react with immune serum. However, Western blots of extracted amastigotes identified several bands not seen on radiographs and thus not accessible to ^{125}I. The external exposure of these amastigote molecules was confirmed in that immune serum adsorbed with viable, intact amastigotes was no longer reactive with amastigote extracts. Further, by Western blot analyses of sodium dodecyl sulfate- but not Nonidet P-40-extracted amastigotes, three amastigote-specific membrane antigens not previously observed with nonionic detergent extraction methods were identified. The autofluorographic pattern of amastigotes intrinsically labeled with *N*-[^{3}H]acetylglucosamine, an amino sugar which is incorporated into membrane carbohydrates, was in excellent agreement with the pattern of antigens reactive with antibody in Western blots. Thus, with these cell surface labeling and extraction methods, promastigote and amastigote membranes were shown to be significantly different. Amastigotes possessed several membrane-associated molecules, but few appeared to be either accessible or reactive with ^{125}I. Moreover, the majority of molecules not reactive with ^{125}I, but reactive with antibodies, may be glycosylated. These observations are discussed relative to the ability of amastigotes both to survive within the degradative milieu of macrophage phagolysosomes and to evade host immune reactivity.

Intracellular and extracellular pathogens presumably possess an array of membrane determinants that elicit protective host immune responses. On most extracellular bacteria and protozoa these immunogens induce antibody production and subsequent antibody-dependent clearance mechanisms which usually result in efficient removal of the pathogen. In contrast, the killing of intramacrophage parasites requires the release of T-cell factors that induce macrophage (Mφ) activation. Importantly, in most cases, if T-cell stimulation and concomitant lymphokine production do not occur, Mφ are unable to destroy the parasite, even in the presence of specific antibody (13, 22, 26).

Leishmania species cause an infection requiring the development of cell-mediated immunity for recovery and protection from reinfection. The flagellated, extracellular promastigote stage gains entry to its mammalian host by the bite of infected sand flies. Although most promastigotes are destroyed after ingestion by resident Mφ (~80%; 21), the remaining organisms convert to resident Mφ-resistant amastigotes and initiate replication within the phagolysosomes.

Intramacrophage parasitism by *Leishmania tropica* provides a model to pursue questions pertaining to (i) the differences between promastigotes and amastigotes that re-

sult in the relative promastigote susceptibility and amastigote resistance to Mφ-mediated killing and (ii) the apparent inability of susceptible hosts to mount the immune response necessary to activate Mφ and destroy the amastigotes. Although host responses to infection have been identified as one crucial factor determining the outcome of infection, parasite-specific properties such as immunosuppressive factors (6) may also contribute to their ability to evade host recognition and thereby propagate within Mφ. As an alternative survival mechanism, we have begun to investigate parasite membrane components for their protective role. Observations from other laboratories suggest that both promastigotes and amastigotes possess surface carbohydrates that may serve as cell adhesion molecules. Such external carbohydrates may decrease the accessibility to membrane proteins (5, 10). Monoclonal antibodies to promastigotes and amastigotes identified peptide epitopes that may be shielded by carbohydrates (10). Moreover, it has also been demonstrated that amastigote membrane proteins are resistant to in vitro proteolysis by papain, chymotrypsin, and staphylococcal V8 protease (12). These data suggest that fundamental differences exist between *L. tropica* promastigote and amastigote membrane composition and that further biochemical analysis might provide information relevant to the intra-Mφ survival of *L. tropica* amastigotes.

In this report we used surface and metabolic radiolabeling and polyacrylamide gel electrophoresis to examine differ-

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ences between promastigote and amastigote membranes. Data are provided that suggest that most amastigote cell membrane antigens are glycosylated. Further, amastigotes, as compared with promastigotes, possess an overall paucity of membrane determinants. The relationship of these findings to the immunology of leishmaniasis is discussed.

MATERIALS AND METHODS

Leishmania. *L. tropica*, NIH strain 173, originally isolated from a patient in Iran with Oriental sore, was generously provided by R. Locksley. To propagate amastigotes in vivo, BALB/c mice (6 to 8 weeks, bred in-house) were injected subcutaneously in both hind footpads with 10^5 to 10^6 viable amastigotes. Amastigote suspensions were prepared from infected tissue 3 to 4 weeks later according to the method of Nacy et al. (24). Briefly, mice were killed by cervical dislocation and immersed in ethanol for 20 min. The hind feet were removed and disrupted by passage through a no. 50 mesh stainless-steel screen (Bellco Glass, Inc., Vineland, N.J.) into 37°C RPMI 1640 (GIBCO Laboratories, Long Island, N.Y.) containing 50 μg of gentamicin per ml. The resulting suspension was homogenized in a 50-ml Bellco tissue homogenizer (10 strokes) to release amastigotes from infected macrophages. The homogenized suspension was centrifuged at low speed (200 × *g*) for 10 min at room temperature to remove cell debris. The amastigotes, recovered from the supernatant by centrifugation at 1,800 × *g* for 30 min, were suspended in 45% Percoll (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) and layered over 100% Percoll (3). The gradient was centrifuged at 5,000 × *g* for 50 min, and the amastigotes were recovered from the interface between the 100 and 45% Percoll layers. The amastigotes were washed three times in sterile phosphate-buffered saline (PBS) and quantitated by counting with a known concentration of chicken blood cells, using light microscopy (24).

Promastigotes were generated by culturing 10^8 amastigotes in 300 ml of M199 media (GIBCO) supplemented with 30% heat-inactivated fetal bovine serum (Hy Clone; Sterile Systems, Logan, Utah) and antibiotics. After incubation in a 27°C water bath for 5 days the 300-ml culture contained 4 × 10^9 to 6 × 10^9 promastigotes. Promastigotes were quantitated by counting in a Petroff/Hausser counting chamber, harvested by centrifugation at 1,800 × *g* for 20 min, and washed three times in sterile PBS.

***L. tropica* iodinations and sample preparation.** A total of 2 × 10^9 to 5 × 10^9 viable promastigotes and amastigotes were surface iodinated with ^{125}I (7), using either IODOGEN (Pierce Chemical Co., Rockford, Ill.) (15) or the lactoperoxidase method of Hubbard and Cohn (16). Labeled promastigotes and amastigotes were killed by freeze-thawing and extracted for 30 min at 4°C in 0.2 ml of PBS containing 0.5% Nonidet P-40 (NP40) and 2 mM phenylmethylsulfonyl fluoride. The extracts were centrifuged at 15,600 × *g* for 10 min (Eppendorf Micro Centrifuge, model 5414), and the supernatants were mixed 1:1 with 2× Laemmli sample buffer (19).

Biosynthetic radiolabeling of *L. tropica*. (i) Leucine/methionine. A total of 3 × 10^9 to 4 × 10^9 *L. tropica* promastigotes or amastigotes were suspended in 5 ml of leucine-free RPMI containing 20% dialyzed fetal bovine serum (dialyzed against PBS) in plastic culture tubes (17 by 100 mm). To each tube was added 200 μCi of L-[4,5-^{3}H]leucine (specific activity, 136 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) and 200 μCi of L-[^{35}S]methionine (specific activity, approximately 239 mCi/mmol; Amer-

sham). Promastigotes and amastigotes were incubated at 27 and 37°C, respectively, for 4 h and extracted as above.

(ii) *N*-Acetylglucosamine. A total of 2 × 10^9 *L. tropica* promastigotes or amastigotes were cultured in 5 ml of glucose-free RPMI containing 15% dialyzed fetal bovine serum and 250 μCi of *N*-[^{3}H]acetylglucosamine ([^{3}H]NAG; specific activity, 2.84 Ci/mmol; Amersham). After 8 h of incubation (promastigotes at 27°C and amastigotes at 37°C), the organisms were washed five times in PBS and extracted in either 0.5% NP40 in 10 mM Tris-hydrochloride (pH 7.5) or 1× Laemmli sample buffer containing 3% sodium dodecyl sulfate (SDS) (19).

Gel electrophoresis. Nonidet P40 extracts of radiolabeled or unlabeled organisms were mixed with an equal volume of 2× Laemmli sample buffer and boiled for 3 min. Two to 10 μg of unlabeled protein, 10^5 cpm from biosynthetically labeled cell extracts, or 2 × 10^5 trichloroacetic acid-precipitable cpm from ^{125}I-labeled organism extracts were electrophoresed in each lane of a 15-cm SDS–11% polyacrylamide gel according to the Laemmli procedure (19). Gels of iodinated extracts were dried and exposed to X-ray film with an intensifying screen (Lightning Plus; Dupont Corp., Wilmington, Del.) at −80°C. For autofluorographic of ^{3}H-labeled preparations, methanol/acetic acid-fixed gels were impregnated with Autofluor (National Diagnostics, Summerville, N.J.), dried, and exposed to preflashed X-ray film at −80°C.

Antiserum production and Western blot analyses. A female New Zealand white rabbit was immunized with a mixture of freeze-thawed *L. tropica* promastigotes and amastigotes (1 mg each) in 4 ml of Freund complete adjuvant. The rabbit was boosted with 1 mg each of promastigotes and amastigotes in 4 ml of Freund incomplete adjuvant at several weekly intervals. Blood was collected before each immunization, and the sera were tested by indirect immunofluorescence on viable promastigotes and amastigotes. Serum after the sixth immunization was found to be highly reactive with both *Leishmania* stages and was collected for subsequent experimental use. The serum was precipitated in 40% ammonium sulfate, reconstituted in 5 ml of PBS, dialyzed against two 4-liter changes of PBS, and stored at −20°C. Immune serum, diluted 1:200, was strongly reactive against promastigotes and amastigotes by indirect immunofluorescence. Ammonium sulfate-precipitated normal rabbit serum was nonreactive at 1:100.

Western blot analysis of promastigote and amastigote NP40 and 1× Laemmli sample buffer (19) extracts was performed essentially as previously described (1). The blotted gel was blocked with 1% bovine serum albumin and incubated overnight with a 1:200 dilution of ammonium sulfate-precipitated serum, and the washed nitrocellulose paper was reacted with 5 μg of [^{125}I] protein A (specific activity, 2 to 10 μCi/mg; New England Nuclear, Boston, Mass.). Dried blots were exposed to X-ray film at −80°C for 1 to 4 days with an intensifying screen. Blots reacted with 1:200 normal rabbit serum demonstrated no bands (data not shown). For some experiments, 1.0 ml of a 1:10 dilution of ammonium sulfate-precipitated immune serum was adsorbed twice for 30 min each on ice with 200 μl of packed viable promastigotes or amastigotes. The adsorbed sera were unreactive by indirect immunofluorescence with freshly isolated promastigotes or amastigotes.

RESULTS

Compositional analysis by biosynthetic labeling. Promastigotes and amastigotes were intrinsically labeled with

[³H]leucine and [³⁵S]methionine to identify endogenously synthesized protein molecules (Fig. 1). Both stages (promastigotes, lane A, and amastigotes, lane B) possessed numerous radiolabeled proteins extractable in NP40, the majority of which were of dissimilar molecular weights.

Iodination of promastigotes and amastigotes. To specifically examine those membrane molecules that were externally exposed, viable promastigotes and amastigotes were surface iodinated with ¹²⁵I, using both the lactoperoxidase and the IODOGEN techniques. The corresponding autoradiograph (Fig. 2) shows that both amastigotes (lanes A and B) and promastigotes (lanes C and D) possessed an intensely iodinating moiety at approximately 9,500 daltons (9.5K), detectable with lactoperoxidase (lanes A and C)- and IODOGEN (lanes B and D)-mediated iodination. However, whereas promastigotes possessed multiple iodinating molecules (major bands appearing at approximately 40K, 42K, 43K, 48K, 53K, 58K, 64K, and 71K), the amastigotes iodinated with IODOGEN had two distinct weakly iodinating molecules at approximately 47K and 57K and two barely visible bands at 24K and 35K.

Western blot analysis of antibody-reactive leishmanial molecules. Promastigote and amastigote NP40 and SDS sample buffer extracts were electrophoresed, and the electro-transblotted gels were reacted with a heterologous rabbit antipromastigote/amastigote serum. The migration of antibody-reactive molecules from NP40-extracted promastigotes (Fig. 3, lane A) correlated with the molecular weights of molecules extracted from iodinated promastigotes (Fig. 2, lanes C and D). In addition, SDS extracts reacted with immune serum (Fig. 3, lane B) revealed additional bands not previously detected by nonionic detergent extraction. Western blots of NP40- or SDS-extracted amastigotes only partially agreed with the polyacrylamide gels of IODOGEN-iodinated amastigotes. The IODOGEN-labeled extracts (Fig. 2, lane B) possessed only one heavily iodinating band (9.5K) and two very weak bands (47K and 57K). The NP40 extracts

FIG. 2. Autoradiography of NP40 extracts from iodinated *L. tropica* promastigotes and amastigotes. Extracts in each lane are: A, lactoperoxidase-iodinated amastigotes; B, IODOGEN-iodinated amastigotes; C, lactoperoxidase-iodinated promastigotes; and D, IODOGEN-iodinated promastigotes.

analyzed on Western blots (Fig. 3, lane C) revealed major antibody-reactive molecules at approximately 9.5K, 20K, and 43K and one other minor band at 32K. The SDS extracts (Fig. 3, lane D) contained antibody-reactive molecules at essentially the same molecular weights, but with the addition of three bands at 22K, 25K, and 29K. The intensely iodinating 9.5K molecule (Fig. 2) was also strongly reactive with antibody in Western blots.
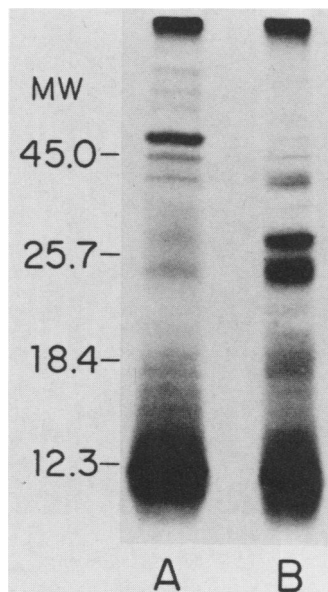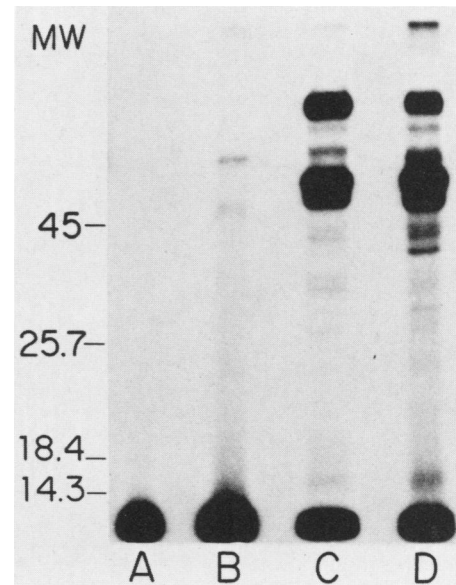

FIG. 1. Autofluorography of NP40 extracts for *L. tropica* promastigotes and amastigotes biosynthetically labeled with [³H]leucine and [³⁵S]methionine. Extracts in each lane are: A, promastigote; B, amastigote.
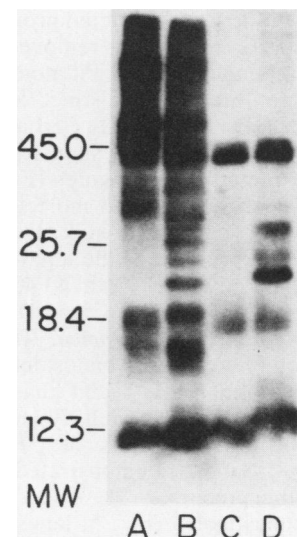

FIG. 3. Western blot analysis of NP40 and SDS extracts from *L. tropica* promastigotes and amastigotes. Blots were developed by autoradiography after interaction with [¹²⁵I]protein A. Extracts in each lane are: A, NP40-extracted promastigotes; B, SDS-extracted promastigotes; C, NP40-extracted amastigotes; D, SDS-extracted amastigotes.
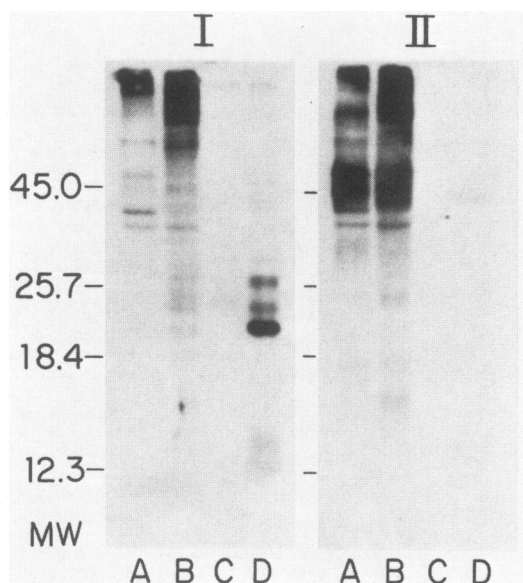
FIG. 4. Western blot analysis by adsorbed immune serum of NP40 and SDS extracts from *L. tropica* promastigotes and amastigotes. The electrotransferred gels were reacted with heterologous rabbit antipromastigote/amastigote sera adsorbed with viable promastigotes (I) or viable amastigotes (II). Blots were developed by autoradiography after interaction with [^{125}I]protein A. Extracts in each lane are: A, NP40-extracted promastigotes; B, SDS-extracted promastigotes; C, NP40-extracted amastigotes; D, SDS-extracted amastigotes.

Western blot analyses using adsorbed antisera. Promastigote/amastigote antiserum adsorbed with either promastigotes or amastigotes were used in Western blot analyses. When promastigote and amastigote NP40 extracts were reacted with promastigote-adsorbed sera (Fig. 4I, lanes A and C, respectively) the reactivity to both stages was generally diminished when compared with the reactivity of unadsorbed serum (Fig. 3, lanes A and C). However, although most reactivity to SDS-extracted promastigotes (Fig. 4I, lane B, versus Fig. 3, lane B) was removed by adsorption (except for a few high-molecular-weight moieties), the activity still remained to three very distinct SDS-extractable amastigote molecules (Fig. 4I, lane D). Serum adsorbed with amastigotes gave reactivity patterns similar to those of both NP40- and SDS-extracted promastigotes (Fig. 4II, lanes A and B). Whereas most reactivity to molecules below 40K was removed, the adsorbed serum still reacted with molecules of >40K. In sharp contrast, the amastigote-adsorbed serum had lost all reactivity to both NP40- and SDS-extracted amastigotes (Fig. 4II, lanes C and D).

[^{3}H]NAG incorporation by *L. tropica*. The discrepancy between the location of amastigote bands detected by Western blots and metabolic labeling and that by iodination prompted the use of [^{3}H]NAG to identify membrane carbohydrates (2, 4). Autofluorographs of [^{3}H]NAG-labeled promastigotes (Fig. 5, lane A) demonstrated few molecules had incorporated the precursor. However, the pattern of [^{3}H]NAG-labeled amastigotes (Fig. 5, lanes C and D) was most comparable to their banding pattern by Western blots. Bands identifiable at 9.5K, 22K, 25K, 30K, 40K, and 44K were common to both gels. When compared with gels of radiolabeled amino acid extracts (Fig. 1, lane B), although intensities were inconsistent, similar patterns were observed at 9.5 to 12K, 15 to 20K, 20 to 30K, and 40 to 52K regions.

Finally, the 9.5K band on gels of iodinated amastigotes was similar in relative intensity to the major antigen incorporating [^{3}H]NAG.

DISCUSSION

These studies compare in detail the external accessibility and expression of *L. tropica* promastigote and amastigote membrane molecules. Other reports have focused primarily on the promastigote stage of various *Leishmania* genera and have essentially confirmed the relatively complex nature of promastigote membranes (4, 7, 8, 21). However, little information is available concerning the membrane constituents of amastigotes, the leishmanial form present in parasitized Mϕ, and perhaps the more relevant stage to human disease. Both Handman et al. (9–12) and Hodgkinson and Patton (13) have identified differences as well as similarities (9–12) between *L. tropica* promastigote and amastigote membranes, although the methodologies used in their experiments are different than those used in ours. The data obtained from experiments comparing the physical properties of promastigotes and amastigotes might provide clues leading to a better understanding of amastigote intra-Mϕ survival.

In the context of host reactivity to pathogenic organisms, externally exposed membrane antigens may, at least initially, contribute to the induction of a cell-mediated immune response. Therefore, the intracellular and extracellular forms were subjected to biochemical analysis to examine differences between external membrane antigens of the two stages. Autoradiographs of NP40 extracts from lactoperoxidase- and IODOGEN-iodinated promastigotes and amastigotes showed marked stage-specific differences in the accessibility of iodinating molecules. Promastigote membranes contained approximately eight heavily iodinating molecules and numerous minor bands. On the other hand, only one predominant molecule (9.5K) was heavily iodinated on amastigotes, with two minor bands (56K and 75K) labeled
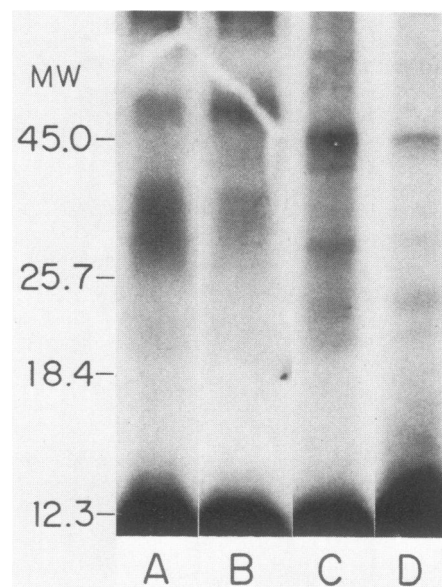

FIG. 5. Autofluorography of NP40 or SDS extracts from *L. tropica* promastigotes or amastigotes biosynthetically labeled with [^{3}H]NAG. Extracts in each lane are: A, NP40-extracted promastigote; B, SDS-extracted promastigote; C, NP40-extracted amastigote; D, SDS-extracted amastigote.

by using the IODOGEN technique. In these experiments, IODOGEN appeared to give a higher labeling efficiency than did lactoperoxidase-mediated iodination. Also, autoradiographs of iodinated promastigotes and amastigotes extracted in SDS were identical to those shown in Fig. 2 (data not shown). The external accessibility of these molecules and the differences between promastigote and amastigote membranes were confirmed by a series of Western blot analyses. With NP40 extracts, and rabbit antipromastigote/amastigote serum, virtually all promastigote molecules accessible to iodination appeared also to be reactive with antibodies. In sharp contrast, Western blot analysis of NP40-extracted amastigotes revealed the presence of at least four new bands. Moreover, antiserum reacted with SDS amastigote extracts identified three additional molecules not seen on NP40 extracts. That these molecules were also external was demonstrated when the antiserum was adsorbed with viable amastigotes and subsequently reacted with transblots of promastigotes and amastigotes. Whereas the amastigote-adsorbed antiserum had a diminished reactivity with several promastigote molecules, all reactivity to amastigote antigens was removed. These experiments strongly suggest that amastigote membranes express approximately eight molecules (Fig. 3, lane D), only one of which is readily accessible to iodination. The Western blot experiments also revealed that amastigotes possess at least three (22K, 25K, and 29K) stage-specific molecules and that these antigens are extractable in SDS, but not NP40. Thus, it appears that although NP40 extracts predominantly membrane constituents, this procedure is too mild for the efficient extraction of some membrane components (2). More importantly, we have identified three membrane antigens, apparently amastigote specific, that may have been missed by using only nonionic detergent extraction.

The discrepancy between autoradiographs of NP40-extracted, ^{125}I-labeled amastigotes and the Western blots of amastigotes was at least partially explained by using the carbohydrate precursor [3H]NAG. This molecule is predominantly incorporated into membranes and does not serve as a carbon source for other endogenously synthesized molecules (2, 4). Autofluorographs of labeled amastigotes demonstrated bands that closely correlated with the molecules detected on Western blots. These data suggest that glycosylation of amastigote membrane antigens may make them inaccessible to iodination or that these membrane constituents may not contain any iodinating molecules.

The composition of the 9.5K molecule resulting in its intense iodination and incorporation of [3H]NAG is intriguing. To explore the consistency of our findings, promastigotes and amastigotes from another *Leishmania* species, *L. mexicana amazonesis* (NIH Maria strain; 28) were iodinated and their extracts were analyzed as above. Again amastigotes possessed only the one 9.5K iodinating molecule (H. Raff and M. Sadick, unpublished data). Moreover, Gardiner et al. (7, 8) observed this iodinating molecule on four leishmanial species. In other studies (Raff and Sadick, unpublished data) amastigotes cultured with [3H]palmitate incorporated large amounts of the fatty acid into the 9.5K molecule. A high fatty acid content could explain the strong reactivity with free iodine. The composition of this molecule is under further investigation in our laboratory.

Other laboratories have investigated the antibody responses to promastigote and amastigote antigens, using sera from infected humans or mice. The sources of the sera used in Western blot analyses and immunoprecipitations varied (immunized rabbits or mice and infected mice [11], infected mice [25], and infected humans [17]), making direct comparison of these data with ours difficult. In general, these studies have indicated a greater degree of cross-reactivity between the antibody responses to the amastigote and promastigote stages of *Leishmania* than was found in our studies. The intent of our study was not to contrast the antileishmanial activity of sera from immunized versus infected animals, but rather to explore the antigen accessibility on the membrane surface of the two stages by using a common antibody source, immune rabbit serum. It is interesting that serum from infected animals may possess quite different antileishmanial reactivity than serum from immunized animals. We used serum from immunized rabbits to maintain an internal consistency and avoid the potential confusion which might occur when sera from disparate sources are used. Whereas serum from the promastigote/amastigote-immunized rabbit did reveal cross-reactivity between some externally exposed membrane molecules on both stages of *L. tropica*, these studies do demonstrate that amastigotes possess some unique membrane antigens.

Since amastigotes are obtained from infected mouse footpads, the possibility had to be excluded that amastigotes were coated with host proteins. Two observations make this unlikely. First, murine spleen cells, directly extracted or iodinated and extracted, and mouse serum were analyzed by gel electrophoresis. The banding patterns did not resemble the pattern of extracted amastigotes (data not shown). Second, electron microscopic analysis has demonstrated that host material attached to freshly isolated amastigotes is shed within 4 h (18, 27). In our experiments amastigotes were incubated at 37°C for 2 to 4 h to allow this release of host-derived material. Moreover, the biosynthetic labeling experiments make it further unlikely that host tissues contributed to our observations.

Whether the differences in the exposure and expression of promastigote and amastigote membrane molecules underlie the host immune reactivity to these two stages of the parasite is unknown. Although it might be assumed that a reduction in membrane antigens would elicit a blunted immune response, and thus a better opportunity for parasite survival, models have not been available to address this issue. In related experiments (M. Sadick and H. Raff, Cell. Immunol., in press) promastigotes were more immunogenic than amastigotes when used in killed antigen preparations with adjuvant. These results were based on secondary in vitro bioassays of T-cell proliferation and interleukin-2 synthesis and supported the hypothesis that amastigotes are but weakly antigenic. A causal relationship between reduced T-cell responses and reduced expression and exposure of amastigote membrane antigens has not been established; however, this possibility should be more fully investigated.

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