Amastigote Stage-Specific Monoclonal Antibodies against *Leishmania major*

CHARLES L. JAFFE* and NURIT RACHAMIM

Department of Biophysics, MacArthur Center for Molecular Biology of Tropical Diseases, Weizmann Institute of Science, Rehovot 76100, Israel

Received 12 May 1989/Accepted 16 August 1989

Monoclonal antibodies were produced against gamma-irradiated amastigotes of *Leishmania major*. Five antibodies (T16 through T20) were selected which reacted in enzyme-linked immunoassays with the intracellular stage of the parasite. These antibodies did not react with promastigotes of *L. major* or *Leishmania donovani*. One of the monoclonal antibodies (T16) reacted with amastigotes of *Leishmania mexicana amazonensis* and *L. donovani*. Western blotting (immunoblotting) and immunoprecipitation of [35S]methionine-labeled amastigotes demonstrates that T16 reacted with multiple *L. major* amastigote components between 12 and 180 kilodaltons. Antibody T20 was shown to recognize a low-molecular-mass doublet (<26 kilodaltons) in both [14C]leucine- and [35S]methionine-labeled amastigotes. A protein of <180 kilodaltons was also weakly recognized by T17, T19, and T20 in metabolically labeled amastigotes. This protein reacted strongly with T16.

The reactive antigens could be identified on the surface of amastigotes isolated from the lesions of infected mice and on newly transformed amastigotes within 24 h after the infection of mouse peritoneal macrophages by promastigotes. These monoclonal antibodies should prove useful for the diagnosis of *L. major* in human tissue biopsies.

Leishmaniasis encompasses a spectrum of diseases caused by the protozoan parasites of the genus *Leishmania* (6, 27, 28). These parasites have a relatively simple digenetic life cycle, existing in the sandfly vector as flagellated promastigotes and in suitable host macrophages as intracellular amastigotes (6, 27, 28). Adaption by the parasite to drastic changes in the external environment during its life cycle results in alterations of metabolism and morphology during differentiation. Because of the relative ease with which the promastigotes can be cultured, extensive studies on the biochemical and immunological characteristics of promastigote antigens have been carried out (1, 6, 27, 28). Fewer studies of a similar nature have been undertaken with amastigotes due to the difficulties encountered in obtaining sufficient cells. However, these studies show that stage-specific components are present on the parasite and that these antigens undergo modulation during parasite differentiation (7, 11, 13, 15, 29).

Monoclonal antibodies (MAbs) have been important for the identification of species- and subspecies-specific determinants on *Leishmania* antigens (3, 10, 23). Promastigote stage-specific MAbs (8, 12, 17; D. McMahon-Pratt, E. Bennett, and C. L. Jaffe, Fed. Proc. 43:1628, 1984) have been used to study parasite differentiation (9, 24; Jaffe and McMahon-Pratt, unpublished results). In addition, MAbs have also been valuable in the purification of species-specific components, such as the promastigote surface protease, 46KDa/M2, lipophosphoglycan, gp70-2, and dp72 (5, 14, 19, 20), from promastigotes for further characterization and immunological studies. Little attempt has been made to produce MAbs specific for the intracellular stage of the parasite. Recently, Pan and McMahon-Pratt (26) showed that an axenic amastigoteline cell line of *Leishmania mexicana pifanoi* could be used to produce amastigote stage-specific MAbs to this *Leishmania* species. Here we report on the production and characterization of amastigote stage-specific MAbs to *L. major* amastigotes isolated from BALB/c mice.

**MATERIALS AND METHODS**

**Production of MAbs.** Female BALB/c mice (Olac, Bicester, United Kingdom) were immunized with enriched membrane preparations (fusion IS-17) and/or intact X-irradiated amastigotes (fusion IS-27) of *L. major* WR309 (MHOM/IL/79/Perlstein). MAbs for immunization were isolated from BALB/c mice as described below. Crude amastigote membrane fractions were prepared by differential centrifugation after parasite disruption by sonication for 40 s at 0°C in an E/MC ultrasonic bath. The amastigotes were suspended in 20 mM Tris buffer (pH 7.3) containing various inhibitors of proteolytic enzymes. The cell homogenates were fractionated as previously reported for promastigotes (17). Approximately, 20 μg of protein from the enriched fraction emulsified with incomplete Freund adjuvant per mouse was injected subcutaneously every 3 weeks. The final boost was carried out 40 days before the fusion by both the intraperitoneal and subcutaneous routes in phosphate-buffered saline (pH 7.2) (PBS).

Mice immunized with irradiated amastigotes (100,000 rads, cobalt gamma) received two injections of 105 parasites, half intraperitoneally and half intravenously, 1 month apart. These mice were given a final boost of amastigote membranes as above 4 days before the fusion. Hybrid cells were produced by fusing NS-1 (P3-X63Ag8/E8) cells (107) with spleen cells (106) isolated from the immunized mice as described previously (23).

**Parasites.** Promastigotes were cultured at 26°C in Schneider *Drosophila* medium supplemented with 10% fetal calf serum. Amastigotes of *L. major* (WR309) and *Leishmania mexicana amazonensis* (MHOM/BR/??/LLT8016) were isolated from lesions on the rear feet of infected BALB/c mice. The parasites were purified on discontinuous Percoll gradients as described by Jaffe et al. (16). Amastigotes of *Leishmania donovani* (MHOM/SN/??/Khartoum) were isolated...
from the spleens of infected GH Syrian hamsters. They were purified by a procedure similar to that used for *L. major* amastigotes with the following modifications. The spleen was washed several times in PBS containing 20 mM EDTA, 550 mM glucose, and 2 mM phenylmethylsulfonyl fluoride, and the tissue was teased apart in this solution. Large debris was allowed to settle out for 5 min, and the volume was adjusted to 30 ml with PBS-EDTA-glucose containing phenylmethylsulfonyl fluoride. Erythrocytes were partially depleted by centrifugation twice for 5 min at 400 rpm. The cells remaining in the final supernatant were pelleted by centrifugation for 15 min at 2,000 rpm and suspended in 8 ml of a 45% Percoll solution in PBS-EDTA-glucose. Occasionally this solution was then passed through a 23-gauge needle. A step gradient was built by overlaying a 70% Percoll solution (1.5 ml) with the amastigotes in 45% Percoll (4 ml), followed by a 25% Percoll solution (4.5 ml). The gradients were centrifuged at 1 h at 5,900 × g, and the amastigotes were removed. The amastigotes banded at the 45%-70% interface just above the erythrocytes, which migrated into 70% Percoll. The parasites were diluted in PBS-EDTA-glucose and pelleted by centrifugation for 20 min at 2,000 rpm. Frequently all species of amastigotes were then passed through the J774.1 macrophage cell line and reisolated on Percoll gradients after 24 h. This was necessary to remove host proteins, such as immunoglobulins, which had bound to the surface of the amastigotes (Jaffe and McMahon-Pratt, unpublished data).

**Transformation in macrophages.** Starch-elicited peritoneal macrophages were isolated from BALB/c mice (25), counted, and plated overnight directly on sterile tissue culture dishes or on sterile glass cover slips preplaced in the wells of 24-well microdilution plates. Nonadherent cells were removed by several washes with RPMI 1640, and a 30:1 excess of recently isolated stationary-phase *L. major* promastigotes (fewer than 10 passages in culture) was added in RPMI 1640 plus 10% fetal calf serum. After 3 h of incubation at 37°C (∆t = 0 h) and again after additional 2 and 4 h, excess promastigotes were removed by washing with RPMI. The infected cells were incubated for 12, 24, 47, 72, or 120 h from ∆t = 0 h. At each time point all of the infected cells were washed with RPMI, and the appropriate glass cover slips were removed and dried for immunofluorescence studies. Cells were also detached from the tissue culture plates by incubation twice for 15 min with trypsin (0.3% in PBS) at room temperature and scraping with a sterile rubber policeman. The cells were washed once with RPMI by centrifugation (15 min, 2,000 rpm), adjusted to 3.5 × 10⁶ cells per ml, and cytocentrifuged onto glass microscope slides for immunofluorescence studies.

**Indirect immunofluorescence.** Air-dried preparations were flooded for 5 min with PBS containing 5% fetal calf serum. The excess solution was decanted off, and the slides were incubated for 30 min with undiluted MAb culture supernatants. After rinsing, the slides were further incubated with 45 µl of a 1:20 dilution of fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin G (Bio-Yeda, Rehovot, Israel). Slides were examined on an Olympus BHS microscope.

**Enzyme-linked immunosorbent assay.** Binding of MAbs to *Leishmania* promastigotes and amastigotes was carried out by using intact parasites bound to polyvinyl chloride U-bottomed microdilution plates (2 × 10⁶/ml). The cells were coupled to poly-L-lysine-coated plates with 0.5% glutaraldehyde as described for mammalian cells by Kennett (21). Plates were generally stored at −20°C. When needed, the plates were thawed, rinsed once, and incubated with 50-µl samples of the MAbs either for 2 h or overnight at 4°C. After removal of excess antibodies by washing (three times) with 0.1% Tween 20 in PBS, an enzyme-linked immunosorbent assay was carried as described by Jaffe and Sarfstein (18). The A₄₀₅ and A₉₀₀ were read on a Bio-Tek EL310 microplate reader (Biotek Instruments, Inc.) after 1 h of incubation with enzyme substrate; binding data are expressed as the A₄₀₅ minus the A₉₀₀ (A₄₀₅ − A₉₀₀). Negative controls (NS-1 culture supernatants) and positive controls (IS-17 test bleed) were included on each plate.

**Metabolic labeling and immunoprecipitation.** Amastigotes were isolated from the lesions of infected BALB/c mice as described above. The parasites were washed extensively in either leucine- or methionine-free RPMI and allowed to reincubate at 37°C in this medium for 20 min. [³⁵S]methionine or [¹⁴C]leucine (0.4 or 0.25 mCi, respectively; Amersham International, England) was added to 10⁶ parasites and incubated overnight at 37°C. The cells were washed extensively in complete RPMI, solubilized for 1 h on ice with 0.5% Nonidet P-40 (600 µl) in PBS containing proteolytic inhibitors, and prepared for immunoprecipitation as described above with MAbs against promastigote antigens (18), with the following modifications. In brief, the solubilized material was preincubated for 1 h with a negative control ascites fluid (15 µl). This was followed by incubation with rabbit anti-mouse immunoglobulin G bound to protein A-Sepharose 4B (Sigma Chemical Co., St. Louis, Mo.) for 30 min. After removal of the material nonspecifically bound to the protein A resin, ascites fluids (2 µl) containing the antibodies to be examined were incubated overnight at 4°C with the radiolabeled supernatant, and the immune complexes were removed on protein A-Sepharose 4B precoated with rabbit anti-mouse immunoglobulin (BioMakor, Rehovot Israel). The resin was then washed by centrifugation and washed several times with 0.1% Nonidet P-40 and 0.1% Nonidet P-40/0.35% sodium deoxycholate in PBS before boiling in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer for analysis on 10% polyacrylamide gels (22).

**RESULTS**

**Specificity of MAbs.** Culture supernatants from five fusions were screened by enzyme-linked immunosorbent assay on glutaraldehyde-fixed promastigotes and amastigotes of *L. major*. MAbs produced by five hybridoma cells (Table 1) were selected for further characterization. All of the antibodies examined, IS-17 4B12-E1 (T16), IS-17 4C1-H3 (T17), IS-27 3D7-B8 (T18), IS-27 3A3-A4 (T19), and IS-27 7A11-C3 (T20), reacted with amastigotes of *L. major*. Antibody T16 showed the strongest binding (A₄₀₅ − A₉₀₀) to amastigotes of this parasite and was the only antibody that also

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**TABLE 1. MAbs specific for *Leishmania* amastigotes**

<table>
<thead>
<tr>
<th>MAbs</th>
<th>Binding (A₄₀₅ − A₉₀₀)</th>
<th><em>L. major</em></th>
<th><em>L. donovani</em></th>
<th><em>L. mexicana</em></th>
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<tr>
<td></td>
<td>A</td>
<td>Pro</td>
<td>A</td>
<td>Pro</td>
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<tr>
<td>T16</td>
<td>0.95</td>
<td>0.02</td>
<td>1.04</td>
<td>0.06</td>
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<tr>
<td>T17</td>
<td>0.32</td>
<td>0.02</td>
<td>0.04</td>
<td>0.01</td>
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<tr>
<td>T18</td>
<td>0.43</td>
<td>0.03</td>
<td>0.03</td>
<td>0.07</td>
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<tr>
<td>T19</td>
<td>0.33</td>
<td>0.01</td>
<td>0.02</td>
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<tr>
<td>T20</td>
<td>0.33</td>
<td>0.04</td>
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* A, amastigotes; Pro, promastigotes; ND, not determined.
FIG. 1. Binding of stage-specific MAbs to promastigotes and amastigotes of *L. major* as shown by indirect immunofluorescence. A’s, Amastigotes isolated from infected BALB/c mice; Pro’s, promastigotes; Pos, positive control, anti-*L. major* serum; Neg, negative control, an *L. donovani* species-specific MAb. T17, T18, and T20 are amastigote-specific MAbs.
reacted with amastigotes of other leishmanial species. T16 reacted with amastigotes of *L. mexicana amazonensis* (A<sub>405-490</sub>, 1.70) and *L. donovani* (A<sub>405-490</sub>, 1.04). The other MAbs bound exclusively to amastigotes of *L. major*, and all four showed comparable levels of binding (A<sub>405-490</sub>): T17, 0.32; T18, 0.43; T19, 0.33; T20, 0.33. No reaction was observed with promastigotes of *L. major* or *L. donovani*.

**Immunofluorescence and kinetics of antigen appearance.** All of the MAbs were screened by indirect immunofluorescence on *L. major* by using both amastigotes isolated from infected mice and promastigotes obtained from culture. The four antibodies (T17 through T20) that reacted were specific for amastigotes (Fig. 1, A's). No binding was observed to promastigotes (Fig. 1, Pro's). Antibodies T18 and T20 gave the strongest fluorescence and appeared to bind to the surface of the amastigotes. The pattern and intensity of binding observed for T19 were identical to those of antibodies T18 and T20 (data not shown). The fluorescence observed with T17 was weak, but the pattern noted, a circular ring, resembled those of the other antibodies. The antigens on amastigotes were sensitive to fixation with either methanol or acetone. Prefixation with either solvent resulted in the complete loss of binding by the MAbs to the parasites. Antibody T16 was not suitable for use in the immunofluorescence assay. The positive control test serum reacted with both amastigotes and promastigotes of *L. major* (Fig. 1, Pos). No reaction with either stage of the parasite was observed with the negative control, an *L. donovani* species-specific MAb, D2 (Fig. 1, Neg) (19).

The kinetics of antigen appearance on the parasites during transformation from promastigote to amastigote was examined. Peritoneal macrophages from BALB/c mice were infected with stationary-phase promastigotes of *L. major*. At different times postinfection the parasites were examined by indirect immunofluorescence with MAbs T17 through T20 and with promastigote stage-specific MAbs T1 and T2 (17). Only the results for antibodies T2 and T20 are shown in Fig. 2 and 3, since the kinetics of expression for each antibody group, promastigote or amastigote specific, were essentially the same. At Δt = 0 h (3 h postinfection), clear staining of the parasites by T2 was visible (Fig. 2A). As yet, no staining by the amastigote-specific antibodies was observed. Similar
results were obtained at $\Delta t = 11$ h (data not shown) and $\Delta t = 24$ h; staining by T2 (Fig. 2B) was still present and no or only very weak fluorescence was observed with the amastigote stage-specific antibody T20 (Fig. 2E). It should be noted that the fluorescence staining pattern for T2 was changed. Staining no longer covered the surface of the parasite but was observed as bright particles dotting the surface of the infected macrophage. Giemsa-stained slides from $\Delta t = 11$ h showed amastigote-like forms present in the macrophages (data not shown). By $\Delta t = 48$ h, binding of the amastigote-specific MAB T20 was observed (Fig. 2F). A decrease in the intensity of T2 staining was also noted (Fig. 2C). These opposing trends, observed for T2 and T20, continued over the next 4 days. The binding of T2 gradually disappeared, and the reaction of T20 with the amastigotes increased and reached a maximum after about 3 to 5 days of infection. Similar results were obtained when the amastigotes were released from the infected macrophages by trypsin treatment and cytocentrifuged onto slides for IFA (Fig. 3A). At all time points, amastigote bodies could be observed in Giemsa-stained preparations (Fig. 3B).

**Characterization of reactive antigens.** Western blotting (immunoblotting) and metabolic labeling were used in an attempt to identify the antigenic components recognized by the MAbs. Amastigotes isolated directly from animals or after passage overnight through a macrophage cell line gave identical results. Amastigotes were examined by immunoprecipitation of $[^{35}]$methionine-, $[^{14}]$C]leucine-, $[^{3}H]$glucose-, $[^{3}H]$glucosamine-, or $[^{32}]$P$_{i}$-labeled parasites and polyacrylamide gel electrophoresis. No parasite components were identified by T16 through T20 when amastigotes were metabolically labeled with radioactive glucose, glucosamine, or phosphate. However, four of the MAbs examined (T16, T17, T19, and T20) specifically precipitated proteins from amastigotes radiolabeled with methionine or leucine (Fig. 4). The precipitation patterns observed were identical for parasites labeled with either amino acid. The three antibodies (T17, T19, and T20) weakly reacted with the same high-molecular-mass protein (<180 kilodaltons [kDa]; Fig. 4, lanes c through e). In addition, T20 (lane e) strongly reacted with a doublet of approximately 14 kDa. Antibody T16 (lane b) recognized multiple components and reacted strongly with the 14-kDa doublet and the 130- and <180-kDa bands. When these MAbs were examined by immunoblotting, only antibody T16 reacted with amastigotes (data not shown). This antibody reacted with multiple bands ranging in molecular mass from 12 to <180 kDa, similar to the pattern seen with immunoprecipitation. No binding or extremely weak binding to promastigotes of *L. major* was observed.

**DISCUSSION**

*Leishmania* species cycle between the sandfly vector and a mammalian host, differentiating from the promastigote stage in the vector to the amastigote stage in the host and back again to the promastigote in the sandfly (6, 28). Stage-specific changes have been observed for *L. major* and *L. mexicana amazonensis* promastigotes and amastigotes in several limited studies (7, 11, 13, 15, 29) that compared metabolic and cell-surface labeling patterns and immunoprecipitation with polyclonal sera against amastigotes and promastigotes. Most of the amastigote-specific proteins noted appear between 20 and 45 kDa and may be related to small-molecular-mass heat shock proteins, although specific bands at 94 and 120 kDa were also found. A different repertoire of promastigote stage-specific proteins was reported with molecular masses ranging from 26 to 220 kDa.

To date, almost all of the MAbs produced recognize either the promastigote stage or both stages of the parasite (8, 12,
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FIG. 3.

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lesions of infected BALB/c mice as described

in the text and labeled overnight. Lanes: a, total labeling of

amastigotes used for immunoprecipitation; b, T16; c, T17; d, T19; e, T20; f, negative control ascites.

FIG. 4. Immunoprecipitation of [35S]methionine-labeled amasti-
gotes by stage-specific MAbs to Leishmania species. Amastigotes

were isolated from the lesions of infected BALB/c mice as described

in the text and labeled overnight. Lanes: a, total labeling of

amastigotes used for immunoprecipitation; b, T16; c, T17; d, T19; e, T20; f, negative control ascites.

MONOCLONAL ANTIBODIES TO AMASTIGOTES OF L. MAJOR

17; McMahon-Pratt et al., Fed. Proc.). Several of these antibodies

have been shown to protect BALB/c mice against a promas-
tigote challenge (2), and recently mice immunized with either the 46-
or the 68-kDa protein purified from L. mexicana amazonensis were also protected against a challenge of promastigotes (4).

Few MAbs specific to the intracellular amastigote stage have been produced (26; McMahon-Pratt et al., Fed. Proc.),

although this is the stage of the parasite associated with human disease. Since MAbs produced from infected mice

react either with both stages or with only the promastigote stage (12), it has been necessary to utilize purified amasti-
gotes or their cellular fractions to obtain Leishmania MAbs that are specific for the intracellular stage. By using irradi-
ated intact amastigotes and/or crude membrane fractions, we

produced five MAbs that reacted selectively with the intra-
cellular stages of L. major. No reactions with promastigotes

of this species or those from others were observed. One

antibody, T16, also cross-reacted with amastigotes of L.
mexicana amazonensis and L. donovani. This antibody

reacted with multiple components and may recognize a

specific change such as glycosylation, which is associated

with differentiation into the amastigote stage. Changes in

lectin binding between promastigotes and amastigotes of L.
donovani have been reported (32). The intracellular stage

loses its ability to bind peanut agglutinin and acquires

binding sites recognized by wheat germ agglutinin, which

does not react with promastigotes. One MAb, F-4, which is

specific for amastigotes but cross-reacts with both L. mexi-
cana pifanoi and L. mexicana amazonensis, shows a similar

complex recognition of multiple amastigote proteins (26).

The other antibodies, T17 through T20, did not cross-react

with either L. donovani or L. mexicana amazonensis amas-
tigotes. As shown by immunofluorescence, these antibodies

derogained cell surface antigens on amastigotes of L. major

isolated directly from infected mice or after transformation

from promastigotes to amastigotes in vitro. The circular

pattern of cell-surface staining is similar to that observed

with the amastigote-specific MAbs produced against L.
mexicana pifanoi as described by Pan and McMahon-Pratt (26). The appearance of the epitopes recognized by T17 through T20 on the L. major amastigotes lagged behind the morphological transformation of the parasites as seen in Giemsa-stained preparations by light microscopy. Amastigote-like forms were already visible in the infected macrophages at only 6 h postinfection; however, weak positive fluorescence began to appear at approximately 14 to 24 h. This fluorescence increased with time and was strongest at 120 h postinfection, the longest time period examined. The pattern of staining observed is identical for amastigotes isolated from infected animals and those produced by in vitro infections of starch-elicited macrophages.

Antibody T2 is a promastigote stage-specific antibody (17) that has been shown to recognize both membranal and secreted antigens (17, 30). This antibody reacted with lipophosphoglycan (unpublished data) and certain other proteins that probably share a common carbohydrate epitope (17; unpublished data). The binding of this antibody to the parasites disappeared slowly over the time period examined. The fluorescence was strongest immediately after infection of the macrophages with the promastigotes and was considerably less by 48 h and almost gone by 72 h. This corresponds very closely to the kinetics of disappearance of a Leishmania braziliensis panamensis antigen from the surface of infected macrophages in vitro (31). Fluorescence was still visible when the amastigote-like bodies are visible by Giemsa staining, and no promastigotes could be seen; however, the fluorescence did not appear to be associated with the parasites. This may represent either unprocessed dead promastigote or secreted antigen that has not yet been eliminated by the macrophages. This antigen is reexpressed by promastigotes transforming from isolated amastigotes after approximately 18 h (unpublished data), a kinetics very similar to that observed for other promastigote stage-specific antigens (9, 24).

Characterization of the antigens recognized by the remaining MAbS, T17 through T20, was carried out by Western blotting and immunoprecipitation of metabolically labeled amastigotes. Similar proteins were identified from amastigotes labeled with either [14C]leucine or [3H]methionine. Antibody T17 reacted strongly with a doublet of approximately 14 kDa, which may be related to a similar 17.5-kDa–13.5-kDa doublet recognized by the amastigote-specific MAb P-2 in L. mexicana pifanoi (26). In addition T17, T19, and T20 also reacted weakly with a 180-kDa protein. None of these antibodies reacted by Western blotting. The 34- and 43-kDa doublet seen by most of the L. mexicana pifanoi amastigote-specific MAbS (26) was not recognized by any of the L. major MAbS.

These MAbS should prove useful for typing of Leishmania species and direct diagnosis of L. major in touch biopsies. Together with existing promastigote stage-specific antibodies, the amastigote-specific antibodies should prove helpful markers in further studies on parasite differentiation and developmental regulation. This may lead to the identification of potential candidate antigens for vaccine development or drug targeting.

ACKNOWLEDGMENTS

This research was supported in part by the United States-Israel Binational Science Foundation, the John and Catherine T. MacArthur Foundation, the Minerva Foundation, and the Bat-Sheva de Rothschild Fund for the Advancement of Science and Technology.

LITERATURE CITED


