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Surface antigenic change during differentiation of a parasitic protozoan, *Leishmania mexicana*: Identification by monoclonal antibodies

(trypanosomatid flagellates/hybridoma technique/cell differentiation)

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ABSTRACT **The fusion of SP2/0 myeloma cells with spleen cells from mice immunized with *Leishmania mexicana amazonensis* promastigotes produced hybridoma clones. Indirect immunofluorescent antibody assay with live leishmanias showed that the monoclonal antibody 6H12 recognized only the antigens bound to the surface of *L. mexicana amazonensis* promastigotes. It also showed that the antibody bound to neither amastigotes of this species nor to other *Leishmania* species—i.e., *L. braziliensis braziliensis*, *L. tropica*, and *L. donovani*. Monoclonal antibodies from three other clones (4D11, 4H9, and 6A11) were found to compete with 6H12 for binding to *L. mexicana* promastigotes. With lysates of [^{35}S]methionine-labeled promastigotes, all four monoclonal antibodies precipitated the same triplet set of protein bands at the ≈68,000-dalton region, whereas another monoclonal antibody (6G5) precipitated a different band at ≈90,000 daltons. During differentiation of *L. mexicana amazonensis* from amastigotes to promastigotes, there was a 4- to 8-fold increase above the initial level in the binding of 6H12 monoclonal antibody to leishmanias, as detected by enzyme-linked immunosorbent assay and quantitative fluorometric assay, respectively. Thus, we have demonstrated the use of monoclonal antibodies as probes for antigens that change during leishmanial differentiation.**

Many eukaryotic microbes that undergo cell differentiation have provided excellent models for studies in developmental biology. We have reported on the possible use of the digenetic trypanosomatid protozoa as additional models for certain aspects of such investigations (1). Added significance for studying these flagellates is the fact that they can cause diseases in man and domestic animals.

Leishmanias, which represent one group of these organisms, have two developmental stages: the extracellular promastigote in the sandfly gut and the intracellular amastigote in the mammalian macrophages (2). Recent development of better methods for the cultivation of *Leishmania mexicana* in cells of the macrophage line J774G8 (3) and for the purification of amastigotes from such cultures (3) or from the lesions of infected laboratory animals (4) facilitates investigations of leishmanial differentiation beyond morphological studies and simple metabolic experiments (reviewed in ref. 1). Other workers have shown that the proteinases and enzymes of the glycolytic cycle and of the fatty acid metabolism are different between the two stages and that amastigote-to-promastigote differentiation is enhanced by carbon dioxide and fatty acids (5–9). We have studied antigenic and molecular changes during leishmanial differentiation with the ultimate aim of elucidating the molecular basis of developmental regulation in this system. We found induction and reduction of tubulin biosynthesis during amastigote-to-promastigote and promastigote-to-amastigote differentiation, respectively (1). In contrast to many other eukaryote systems, this change in tubulin biosynthesis is apparently under post-transcriptional control (10). We also have identified stage-specific antigens and their changes during intracellular differentiation of leishmanias in macrophages (11).

Using the same culture system, we report here our finding of promastigote-specific surface antigens as identified by monoclonal antibodies (12) and the change of these antigens during amastigote-to-promastigote differentiation *in vitro*.

MATERIALS AND METHODS

Parasites and Cells. Promastigotes of *Leishmania mexicana amazonensis* (3), *L. braziliensis braziliensis* (LUMP 1640-LV64; Brazil, mucocutaneous leishmaniasis), *L. tropica* (K. P. Chang's clinical isolate; Israel, cutaneous leishmaniasis) and *L. donovani* (1S; Sudan, visceral leishmaniasis) were grown at 27°C in Hepes-buffered medium 199 supplemented with 10% heat-inactivated fetal bovine serum. For *L. mexicana amazonensis*, promastigotes grown in a serum-free defined medium RE (13) were also used. Amastigotes of *L. mexicana amazonensis* were grown at 35°C in a murine macrophage line J774G8 *in vitro* culture system (3). Procedures for amastigote isolation and amastigote-to-promastigote differentiation have been described (1, 3).

The parental cell line used for hybridoma production was SP2/0-Ag 14, a nonproducer, nonsecretor myeloma line (14). The cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM pyruvate, 2 mM glutamine, 50 μM 2-mercaptoethanol, and penicillin/streptomycin antibiotics.

Production of Monoclonal Antibodies. For immunization, male BALB/c mice were injected through the tail vein with 5×10^7 live promastigotes of *L. mexicana amazonensis* in 0.2 ml of Hanks' balanced salt solution. Injected mice subsequently received three inoculations of 2×10^7 promastigotes each, at week 2, week 6, and day 4 before sacrifice.

Fusion between SP2/0 cells and spleen cells of immunized mice was performed by published procedures (15–18). After the lysis of erythrocytes, 10^8 spleen cells were fused with 2×10^7 myeloma cells in 0.5 ml of 30% (vol/vol) polyethylene glycol (PEG). The PEG solution was made by mixing 3 ml of melted PEG (PEG 4000 GK, Merck; see ref. 16) and 7 ml of serum-free hybridoma medium (15), and the solution was filter-sterilized after adjusting the pH to 7.9. The cells were allowed to fuse for 3 min at room temperature and centrifuged for 5 min to remove excess PEG. The cell pellet was gently resuspended in 5 ml of

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; IFA, indirect immunofluorescent antibody assay; P_i/NaCl, phosphate-buffered saline; PEG, polyethylene glycol.

serum-free RPMI medium to which 5 ml of RPMI medium containing 20% serum was subsequently added. After centrifugation, the cell pellet was finally suspended in 30 ml of hybridoma medium containing 20% serum and hypoxanthine/aminopterin/thymidine (HAT). The cell suspension was distributed into six 96-well flat-bottomed culture plates (Costar, Cambridge, MA). To enhance hybridoma growth, a feeder layer of BALB/c mice peritoneal macrophages (at 3×10^3 cells per well) was prepared on the previous day (16). The culture plates were incubated at 37°C in humidified air containing 5% CO_2. Medium was changed from hypoxanthine/aminopterin/thymidine (for a week) to hypoxanthine/thymidine (for a week) and finally to hybridoma medium (for the subsequent weeks). Cell colonies were seen at weeks 2 and 3 in successful fusion experiments.

Enzyme-linked immunosorbent assay (ELISA) for screening anti-leishmania antibodies was performed by using culture supernatants from wells containing colonies. Desired colonies were cloned by the limiting-dilution method in 96-well plates containing macrophages. Hybridomas were expanded in 24-well plates (Linbro) and culture flasks (Falcon). BALB/c mice pretreated with pristane (0.5 ml per animal; Aldrich) were injected intraperitoneally with hybridoma cells (10^7 cells) to produce ascites fluids. Hybridomas were suspended at 10^7 cells per ml of serum containing 5% dimethyl sulfoxide and cryopreserved in liquid nitrogen.

Isotype Analysis. The isotypes of immunoglobulins secreted by hybridomas were determined by Ouchterlony analysis (19). Ten-fold-concentrated culture supernatants were used against class- and subclass-specific rabbit antisera (rabbit anti-mouse IgM, IgG1, IgG2a, IgG2b, and IgG3; Litton Bionetics) in an agar immunodiffusion system (Hyland, Deerfield, IL).

ELISA. ELISA was performed by the method of Engvall (20). Ninety-six-well microtiter plates (Vangard, Neptune, NJ; Nunc-Immunoplate) were coated with poly (L-lysine) (21); live leishmanias (10^8 cells per ml in P_i/NaCl) were added to 50 μl per well. The plate was incubated at 4°C for 60 min to allow the cells to settle and then was submerged gently into P_i/NaCl containing glutaraldehyde (0.5%; Polysciences, Warrington, PA) for 15 min (22). After washings in P_i/NaCl containing 0.25% Tween 20 and 0.02% azide, the plates were stored in P_i/NaCl containing 0.5% gelatin and azide. Culture supernatants or ascites fluids at various dilutions were added into the wells and incubated at 37°C for 3 hr in a humid chamber or at 4°C overnight. These antigen–antibody-coated wells were further processed by regular or sandwich ELISA. For the regular ELISA, peroxidase-conjugated sheep anti-mouse IgG (0.2%; Cappel Laboratories, Cochranville, PA) in P_i/NaCl containing 0.1% bovine serum albumin was added at 100 μl per well and incubated at 37°C for 3 hr. For the sandwich ELISA, rabbit anti-mouse IgG (0.1%; Cappel Laboratories) in P_i/NaCl containing bovine serum albumin was similarly added, incubated at 37°C for 2 hr, and washed; then, peroxidase-conjugated protein A (0.1%; Sigma) in P_i/NaCl/albumin was added, and the mixture was incubated at 37°C for 60 min. The final substrate used for both ELISAs was 2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid) (0.05%; Boehringer Mannheim) in 0.1 M citrate/phosphate buffer, pH 5, containing H_2O_2 (0.05%, from a 30% stock solution). Into each well, 200 μl of the substrate solution was added. After incubation at 37°C for 30 min, reactions were either scored visually or read in a Multiscan (Flow Laboratories, McLean, VA) for colorimetric quantitation.

Indirect Immunofluorescent Antibody Assay (IFA). IFA was adapted from the description by Bray (23). All steps were carried out by using cell suspensions in microcentrifuge tubes and a Microfuge (Beckman) for cell sedimentation. Both live and glutaraldehyde-fixed (as in the ELISA procedure) leishmanias were used. Cells were first treated with culture supernatants or ascites fluids at various dilutions at 2°C for 60 min, washed with P_i/NaCl containing fetal bovine serum (5%), and then treated with fluorescein-conjugated rabbit IgG fraction anti-mouse IgG (10%; Cappel Laboratories) in P_i/NaCl containing serum at 2°C for 30 min. The second antibody was always centrifuged at 39,000 × *g* (Sorvall RC5 Centrifuge, SS-34 rotor) at 4°C for 30 min immediately before use. For fluorescent microscopy, glutaraldehyde-fixed cells were suspended in *p*-phenylenediamine (0.1%; Aldrich) to reduce the fading of fluorescence during photography (24). For quantitation, fluorescent levels of leishmanias were determined by using a Hitachi Perkin-Elmer MPF-4 fluorometer at 495 nm for excitation and 519 nm for emission (25).

Binding Inhibition Assay. Binding inhibition study was adapted from the procedures of Galfre, Milstein, and Wright (26) and Lampson (27). One hybridoma, 6H12, was labeled with [^{35}S]methionine (100 μCi, Amersham; 1,165 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) in methionine-free RPMI 1640 medium (10^7 cells in 5 ml, GIBCO) for 20 hr. The culture supernatant was collected and extensively dialyzed against 2 liters of P_i/NaCl to remove free [^{35}S]methionine. The retentate was used as the source of labeled monoclonal antibody.

Promastigotes of *L. mexicana amazonensis* were glutaraldehyde-fixed (as in the ELISA procedure) onto 96-well flat-bottomed polyvinyl chloride assay plates (Costar). To each microtiter well, 50 μl of a serial dilution of hybridoma supernatants was added. After incubation at 37°C for 2 hr, each well received 10 μl of [^{35}S]methionine-labeled 6H12 monoclonal antibody. The plates were again incubated at 37°C for 2 hr, and the wells were washed in P_i/NaCl containing serum. Individual wells were cut out, and the amount of bound radioactivity was determined by liquid scintillation counting.

Immunoprecipitation of Antigens. The immunoprecipitation steps were the same as described (1) with two modifications. The lysis buffer was 1% (wt/vol) Nonidet P-40 in P_i/NaCl containing 100 μM tosyllysine chloromethyl ketone. Instead of autoradiography, fluorography was used by treating slab gels with Autofluor (National Diagnostics, Somerville, NJ).

RESULTS

Identification of *Leishmania*-Specific Monoclonal Antibodies. With the regular ELISA, culture supernatants were screened for monoclonal antibodies against *L. mexicana amazonensis* promastigotes. Five ELISA-positive wells were selected; the hybridoma cells (4D11, 4H9, 6A11, 6G5, and 6H12) were cloned and expanded as ascites tumors. As a control, cells (4G11) from an ELISA-negative well were similarly cloned and expanded. All five hybridomas, except 4G11, also were found to be IFA-positive for *L. mexicana amazonensis* promastigotes.

One of the hybridomas, 6H12, was selected for further analysis. The titers of the culture supernatant and the ascites fluid were determined by the sandwich ELISA to be 160 and >10,240, respectively. When glutaraldehyde-fixed promastigotes of *L. mexicana amazonensis* were treated with a 1:200 dilution of 6H12 ascites fluid in IFA, uniform fluorescence was seen over the whole cell surface (Fig. 1 *A* and *B*). Under the same condition of treatment, live promastigotes gave a patchy appearance of fluorescence (Fig. 1 *C* and *D*), presumably due to the phenomenon of capping of the surface antigens. A small proportion of promastigotes did not fluoresce under both conditions. Identical results were obtained with promastigotes cultured in the RE defined medium (data not shown), indicating that the monoclonal antibodies were specific to leishmanial cell surface, but not to serum components adherent to the promastigotes.

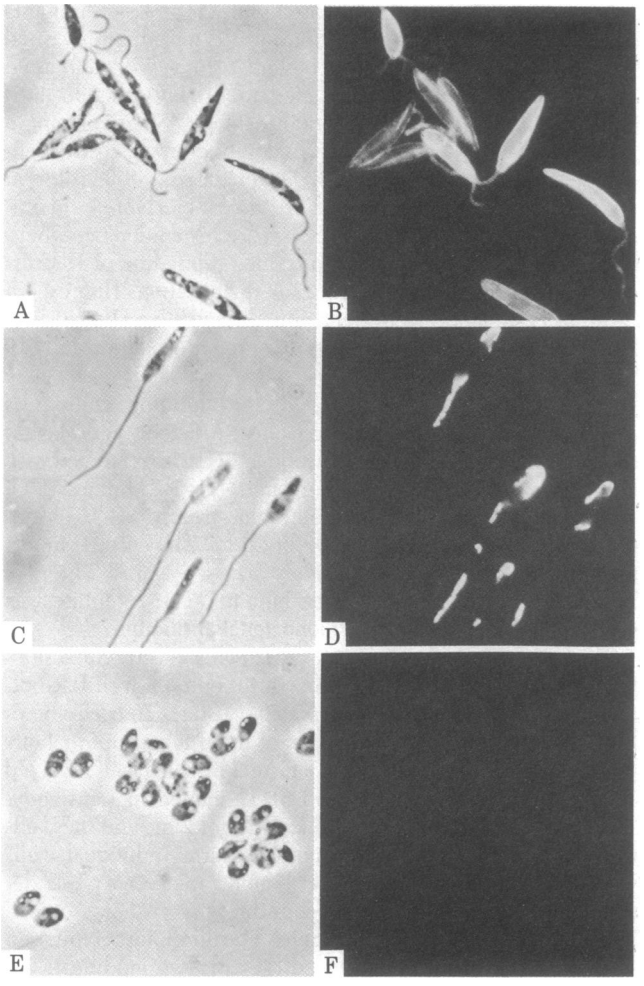

FIG. 1. IFA of *L. mexicana amazonensis* with hybridoma 6H12 ascites fluid at 1:200 dilution. (*A*) Glutaraldehyde-treated promastigotes, phase contrast. (*B*) Immunofluorescence of *A*; (*C*) Live promastigotes, phase contrast. (*D*) Immunofluorescence of *C*. (*E*) Live amastigotes, phase contrast. (*F*) Immunofluorescence of *E*.

The 6H12 hybridoma was stage and species specific. At a 1:200 dilution of the ascites fluid, live *L. mexicana amazonensis* amastigotes were IFA negative (Fig. 1 *E* and *F*). Likewise, 6H12 did not bind to live promastigotes of *L. braziliensis braziliensis*, *L. tropica*, or *L. donovani* (data not shown), even at a 1:10 dilution of the ascites fluid.

In an immunodiffusion system with subclass-specific antisera, 6H12 and three others, 4D11, 4H9, and 6A11, were shown to be IgG1 subclass, whereas 6G5 was probably IgG2b (data not shown). In the binding inhibition study, unlabeled culture supernatants of 6H12, 4D11, 4H9, and 6A11 all competed efficiently with [^{35}S]methionine-labeled 6H12 for binding to *L. mexicana amazonensis* promastigotes, whereas those of 6G5 and 4G11 did not (Fig. 2). Thus, 6H12 and the other three competing monoclonal antibodies probably recognized the same antigenic site.

Immunoprecipitation of Leishmanial Surface Antigens. In order to determine the molecular property of antigens that bound 6H12 and the other monoclonal antibodies, lysates of [^{35}S]methionine-labeled *L. mexicana amazonensis* promastigotes were immunoprecipitated with hybridoma culture supernatants or ascites fluids. Analysis of the immune complexes by NaDodSO$_4$/polyacrylamide gel electrophoresis and fluorography showed that hybridomas 4D11, 4H9, 6A11, and 6H12 all immunoprecipitated the same protein bands—namely, a set

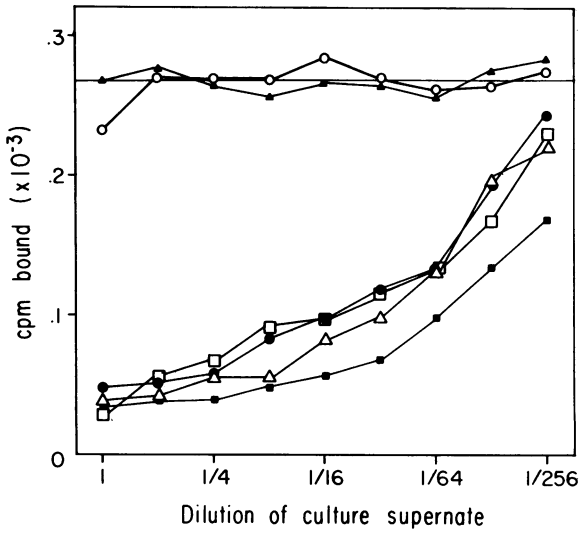

FIG. 2. Binding inhibition assay of monoclonal antibodies. Serial dilutions of unlabeled hybridoma supernatants were used to compete with a fixed amount of metabolically [^{35}S]methionine-labeled 6H12 for the binding to glutaraldehyde-treated *L. mexicana amazonensis* promastigotes. The hybridoma supernatants were 4D11 (■), 4G11 (▲), 4H9 (□), 6A11 (●), 6G5 (○), and 6H12 (△).

of triplets at the 68,000-dalton region; 6G5 precipitated a band of higher molecular mass at 90,000 daltons (Fig. 3). No band was apparent after immunoprecipitation with 4G11 (Fig. 3). The immunoprecipitation experiments agreed well with the binding inhibition assay (Fig. 2) in that both indicated the identity of 4D11, 4H9, 6A11, and 6H12, differing from 4G11 and 6G5. No obvious band was detected by using 6H12 in attempts to immunoprecipitate antigens with [^{35}S]methionine-labeled amastigote extract (data not shown), consistent with the lack of fluorescence on amastigote surface by IFA (Fig. 1).

Antigenic Changes During Leishmanial Differentiation. During amastigote-to-promastigote differentiation at 27°C, there was an emergence and an increase in the binding of 6H12 monoclonal antibody to leishmanias (at 1:200 dilution of the ascites fluid). The binding began to appear after 16 hr of differentiation, coinciding with the emergence of promastigotes. By 40 hr, a 4- to 8-fold increase of the binding above the initial values was noted by the sandwich ELISA (Fig. 4 *Lower*) and the fluorometric assay (Fig. 4 *Upper*), respectively. However, the binding of 4G11 to leishmanias did not increase during this differentiation (Fig. 4 *Lower*). Likewise, the binding of 6H12 to leishmanias did not increase when amastigotes were incubated under nondifferentiation conditions at 35°C for 24 hr (data not shown).

DISCUSSION

In the present study, we have successfully produced hybridoma-derived monoclonal antibodies to *L. mexicana amazonensis*. These monoclonal antibodies are specific to surface antigens of leishmanias, as shown by IFA with live cells and by ELISA with cells mildly fixed with glutaraldehyde. Immunoprecipitation of [^{35}S]methionine-labeled and Nonidet P-40-lysed promastigotes by monoclonal antibodies, followed by NaDodSO$_4$/polyacrylamide gel electrophoresis and fluorography, revealed two sets of surface antigens—i.e., a triplet protein band at 68,000 daltons and a single band at 90,000 daltons. In our previous experiments, we have demonstrated bands of similar molecular mass at 68,000 daltons from *L. mexicana amazonensis* promastigotes by cell-surface iodination, by affinity binding of

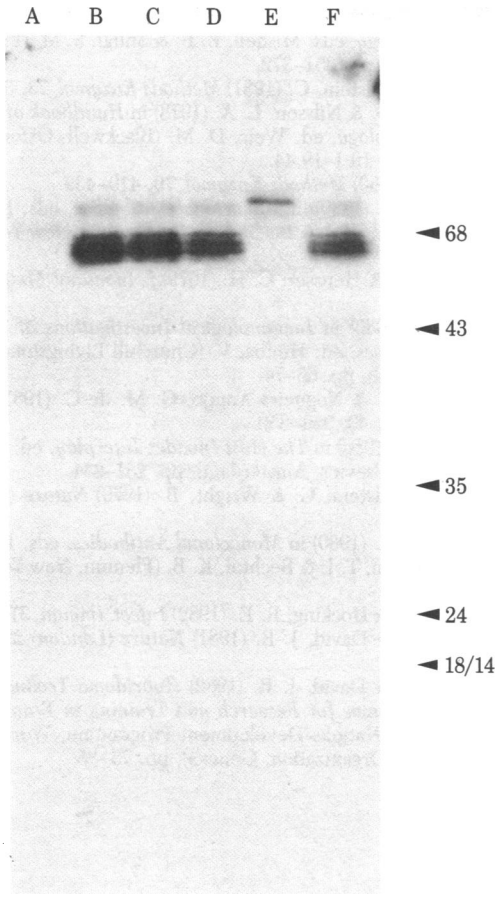

FIG. 3. Immunoprecipitation of the metabolically [^{35}S]methionine-labeled promastigote lysate of *L. mexicana amazonensis* by different hybridoma culture supernatants. Lanes: A, 4G11; B, 4D11; C, 4H9; D, 6A11; E, 6G5; F, 6H12. The immunoprecipitated triplet bands at 68,000 daltons (lanes B, C, D, and F) and the heavy band at 90,000 daltons (lane E) were always reproducible. The triplets may represent precursor–products such as the same polypeptide with various levels of glycosylation, or they may represent the different subunits of a trimeric protein after disulfide band cleavage. The faint bands in other locations are considered as contaminants because they are not reproducible. The molecular mass markers (shown × 10^{-3}) were bovine serum albumin (68,000), ovalbumin (43,000), pepsin (35,000), trypsinogen (24,000), β-lactoglobulin (18,000), and lysozyme (14,000).

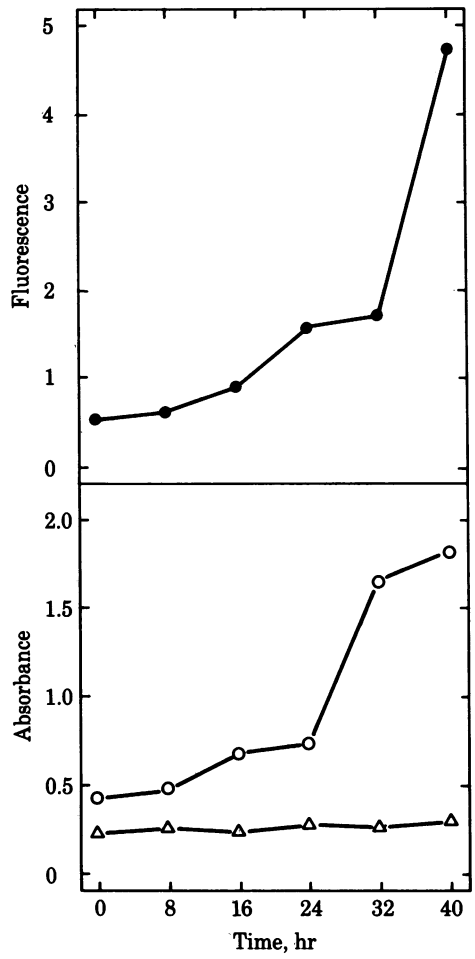

FIG. 4. Antigenic changes during amastigote-to-promastigote differentiation of *L. mexicana amazonensis*. (*Upper*) Binding of differentiating leishmanias to hybridoma 6H12 (●) ascites fluid at 1:200 dilution, as quantitated by fluorometric assay for fluorescein. (*Lower*) Binding of differentiating leishmania to hybridoma 6H12 (○) and the control, 4G11 (△), ascites fluids at 1:200 dilution, as quantitated by sandwich ELISA.

cell extract to concanavalin A-Sepharose 4B, and by immunoprecipitation of cell surface with hyperimmune mouse sera (10). Immunoprecipitation of the same triplet glycoprotein bands by monoclonal antibodies would suggest that they may be the predominant antigens on the surface of this species. In the same study, the only other additional surface antigen detected was a band with an apparent molecular mass of 43,000 daltons (10). The possibility that this antigen may be the monomers of the 90,000-dalton band recognized by hybridoma 6G5 in the present study seems unlikely, but further study is needed. It is more likely that the latter represents a minor surface antigen of *L. mexicana amazonensis* not readily detectable by conventional biochemical and immunological means. All monoclonal antibodies produced in the present work are promastigote-specific because they do not react with amastigotes, as shown by immunofluorescent microscopy and immunoprecipitation experiments. This is further demonstrated by the emergence and increase of the antigen recognized and quantitated by the hybridoma 6H12 during amastigote-to-promastigote differentiation but not under nondifferentiation conditions. A stage-specific monoclonal antibody has been described also for *L. tropica* promastigotes (28).

We also have found that all the monoclonal antibodies produced are specific only to *L. mexicana amazonensis*, as none reacts with *L. braziliensis braziliensis*, *L. tropica*, and *L. donovani* in the IFA. This result supports the finding that *L. mexicana* and *L. braziliensis* can be differentiated by using species-specific monoclonal antibodies (29, 30). Monoclonal antibodies produced against *L. tropica* have been used as immunological reagents to probe the interactions between surface antigens of this species and macrophages; promastigotes treated with some of these monoclonal antibodies fail to grow intracellularly (28). What antigens are recognized by these monoclonal antibodies, however, have not yet been determined.

We have demonstrated here the value of monoclonal antibodies as probes for the identification of surface molecules during leishmanial differentiation. Functional roles of these antigens during differentiation deserve further investigation.

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