INFECTION AND IMMUNITY, Aug. 1982, p. 558-567
0019-9567/82/080558-10$02.00/0

Vol. 37, No. 2

Cell Surface Origin of Antigens Shed by *Leishmania donovani* During Growth in Axenic Culture

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Received 25 January 1982/Accepted 23 April 1982

Antisera against isolated cell surface preparations (PCSP-As) of *Leishmania donovani* promastigotes were used to detect extracellular antigens produced during the growth of these organisms in four different growth media. The PCSP-As precipitated two major antigenically identical but electrophoretically distinct components, in addition to several minor antigens. Immunoelectrophoretic studies employing PCSP-As, PCSP-As absorbed with intact, live promastigotes, and PCSP-As absorbed with a major extracellular antigen demonstrated the antigenic identity between the major extracellular antigens and two major components externally disposed at the surface of promastigotes. Growth curve kinetic investigations suggested that the major extracellular antigens did not appear in the growth media primarily as a result of cell lysis or damage. The carbohydrate nature of the major extracellular antigens was indicated by physicochemical characterization.

Leishmania donovani, a parasitic protozoan, is the etiological agent of kala azar, a chronic, and usually fatal if untreated, form of human visceral leishmaniasis. This organism has a digenetic life cycle, assuming an extracellular, flagellated promastigote form in the alimentary tract of its sandfly vector, and an obligate intracellular form within the phago-lysosomal system of spleen, liver, and bone marrow macrophages of its definitive mammalian hosts (3, 5, 11).

Promastigote forms of most human leishmanial species are readily cultivable in vitro in various serum-supplemented tissue culture media (19) or in several chemically defined *Leishmania* growth media (27, 28). Using antisera made against whole organisms, various investigators working with several different human leishmanial species (i.e., *L. donovani*, *L. tropica*, and *L. braziliensis*) have reported that during promastigote growth or maintenance in vitro, antigens are released from the parasite into the culture media. These in vitro promastigote-released factors have been designated in various reports as leishmanial exogenous, excretory or excreted factors (EF, 15, 20, 24–26); antigenically active glycoproteins (7); and exometabolites (21, 22). Preparations of such promastigote factors have been used as skin test antigens in the diagnosis of patients with cutaneous leishmaniasis and Chagas' disease (6, 23), as well as antigens in the serotyping and classification of *Leishmania* strains and species isolated from patients with active diseases and from insect vectors (20). Further, it has been suggested that such factors have a role in initiating, establishing, and maintaining parasite infections in susceptible and nonsusceptible host macrophages (14, 18, 25). To date, however, despite their apparent relevance to the disease, neither the exact cellular origin nor the definitive chemical composition and structure of these parasite-generated extracellular factors have been unequivocally established.

In the course of studies concerning the structural, biochemical, and antigenic nature of the leishmanial cell surface, a variety of antisera were generated in rabbits against isolated and purified *L. donovani* promastigote cell surface preparations (PCSP), i.e., surface membranes with subpellicular microtubules attached to their cytoplasmic lamina (2, 13). These antisera were used in the current report to establish the relationship between several antigens released by promastigotes during their growth in vitro and those antigens present at the cell surface.

MATERIALS AND METHODS

Organism and growth conditions. A cloned line of *L. donovani* strain 1-S promastigotes (9) was grown at 26°C in four different media: (i) Schneider insect tissue culture medium supplemented with 30% (vol/vol) fetal bovine serum (Schneider + FBS) (4); (ii) medium 199 (GIBCO Laboratories, Grand Island, N.Y.; GIBCO)

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supplemented with 20 or 25% (vol/vol) FBS (199 + FBS) (10); (iii) the chemically defined medium, RE III, formulated by Steiger and Steiger (28); and (iv) a modification of the RE III medium that lacked bovine serum albumin (RE III − BSA) (27).

Preparation of used growth media and fractions. Used growth media were prepared from late-log- or early-stationary-phase cultures, unless otherwise noted. The cell-free culture fluids were obtained after the removal of cells by centrifugation (e.g., large batch cultures were centrifuged in 1-liter bottles at 5,000 × *g* for 30 min at 4°C) and subsequent vacuum filtration through a 0.45-μm porosity membrane filter (type HA; Millipore Corp., Bedford, Mass.).

For studies on changes throughout the growth cycle, *L. donovani* promastigotes were grown in 15 ml of the RE III or the RE III − BSA medium in 50-ml plastic culture flasks (Costar, Cambridge, Mass.) at 26°C. Cells were also grown in 150 ml of RE III or 199 + FBS in 650-ml plastic culture flasks (Costar), and 5 to 7 ml from those larger cultures were removed daily. These small quantities of used growth media were separated from cells directly by filtration through a membrane (Millex, HA 0.45 μm) filter and were stored at −70°C until analyzed by immunoelectrophoresis (see below).

Portions of the used culture media were concentrated approximately 100-fold (100× concentrate), using a pressure dialysis membrane filtration apparatus with a PM-10 membrane filter (Amicon Corp., Lexington, Mass.). To minimize nonspecific binding of concentrated materials to chambers and filters, these were treated with Bacitracin (Calbiochem-Behring Corp., San Diego, Calif.) at 1 mg of distilled water per ml before use.

Physical and chemical treatment of used growth media. For some experiments the 100× concentrate was extracted twice with an equal volume of water-saturated phenol for 1 h at 4°C (16). The aqueous phase was separated and dialyzed exhaustively against 3× 4-liter changes of distilled water at 4°C over 48 h and subsequently lyophilized.

For other experiments a crude carbohydrate (CHO) fraction was obtained by the following protocol: 4 volumes of 95% ethanol was added to the 100× concentrate, and the mixture was stored at −20°C for at least 3 h. After storage, the precipitate formed was recovered by centrifugation at 5,000 × *g* for 15 min at 4°C. The resulting pellet was dissolved in 2% (wt/vol) potassium acetate before reprecipitation with 4 volumes of ethanol at −20°C for at least 3 h. The second ethanol precipitate was recovered by centrifugation and dissolved in distilled water; trichloroacetic acid (TCA) was added to 10 or 33% (wt/vol) final concentration, and the mixture was allowed to stand for 30 min at 4°C. After removal of TCA-insoluble material by centrifugation as described above, the supernatant was concentrated by Amicon PM-10 membrane ultrafiltration and dialyzed overnight at 4°C against 3× 1-liter changes of distilled water, or 0.01 M phosphate-buffered 0.85% (wt/vol) NaCl (PBS, pH 7.4) and containing 15 mM NaN_3.

In some cases the used growth media were further fractionated by passage of the 100× concentrate or the TCA-soluble fraction through an Amicon XM-50 membrane via ultrafiltration. The ultrafiltrate of the 100× concentrate was designated as the ≥10≤50 (10- to 50-) kilodalton (kd) fraction, and the ultrafiltrate of the TCA-soluble fraction was designated the 10- to 50-kd CHO fraction. The retentate was washed twice with 2 chamber-full volumes of distilled water and was designated the ≥50 kd fraction.

The used growth media were also treated with heat, proteolytic enzymes, and sodium meta-periodate. For heat treatment, the samples were placed in a boiling water bath for various periods of time up to 1 h. Proteolytic digestion was accomplished by the addition of pronase (B grade; Calbiochem) to a final concentration of 1 mg/ml (wt/vol) to the 100× concentrate, which had been previously diluted 1:10 (vol/vol) with 0.1 M potassium phosphate buffer adjusted to pH 7.0. The mixture was incubated for 1 h at 42°C. Controls lacking pronase were treated identically. After incubation, the samples were heated in a boiling water bath for 3 min to inactivate the enzyme. Pronase activity was determined by incubation with an artificial substrate (Azocoll; Calbiochem) under identical conditions. Sodium metaperiodate (Sigma Chemical Co., St. Louis, Mo.) was added to the 100× concentrate at a final concentration of 25 mM. Solutions were incubated for 1 h at room temperature and dialyzed overnight at 4°C against 2× 4-liter changes of distilled water to remove the periodate. Controls lacking periodate were prepared identically.

PCSP and antisera. PCSP were purified on sucrose gradients as previously described (12, 17). Identification of the PCSP fraction was determined ultrastructurally by the presence of a homogeneous array of membranes associated with subtending microtubules. The purity of that fraction was judged by the absence of other cellular organelles (12, 17). Triton X-100 (Sigma) extracts of these preparations (13) were used for immunoreactions. Antibodies (PCSP-As) directed against isolated PCSP were raised in New Zealand white rabbits (2, 13). Ammonium sulfate-derived globulin fractions of those sera were concentrated to one third of the original volume (3× concentrate). Alternatively, immunoglobulin G (IgG) fractions were isolated and purified from these sera via protein A-Sepharose CL 4B (Pharmacia Fine Chemicals, Piscataway, N.J.) column chromatography. The latter were concentrated to 30 mg of protein per ml. Antibody fractions were dialyzed overnight against 1 liter of PBS containing 15 mM NaN_3 and subsequently stored at 4°C.

For some studies, the 3× antibody globulin fraction was absorbed with the 10- to 50-kd CHO fraction of the used chemically defined medium (RE III − BSA). This was accomplished as follows: the 10- to 50-kd CHO fraction was first centrifuged at 4,000 × *g* for 60 min, and the resulting supernatant was mixed with a sample of the 3× globulin fraction and incubated sequentially, at 37°C for 30 min and at 4°C for 30 min. This was repeated with two or more additions of the 10- to 50-kd CHO fraction until the pellet size remained constant as judged by visual observation. The resulting supernatant was concentrated to the original 3× volume and designated as the 10- to 50-kd CHO-absorbed antiserum.

Agglutination of intact promastigotes with PCSP-As. Agglutination assays were used to assess the presence of antibodies in PCSP-As capable of binding to externally disposed cell surface antigens. Washed promastigotes were resuspended to 2.5 × 10^7 cells in PBS, and an equal volume of azide-free 3× PCSP-As globulin

fraction was added. Samples were mixed and incubated at room temperature for 15 to 30 min. Cell agglutinations were determined microscopically. Crude CHO fractions of the used growth media were tested for the ability to inhibit such antibody-mediated agglutination. This was accomplished by the addition of crude CHO fraction to a final concentration of 2.5 mg/ml in the presence of the antibody and cells. Agglutination was assessed under the conditions described above.

Gel immunoprecipitin analyses. Gel immunodiffusion reactions were performed with 0.6% (wt/vol) agarose (Litex, type HSA; Accurate Chemical & Scientific Corp., Westbury, N.Y.) made in PBS containing 15 mM NaN_3 and 1% (vol/vol) Triton X-100. Gels were photographed as previously described (8) or fixed and stained with Coomassie brilliant blue R_{250} (30) and photographed.

Gels used for immunoelectrophoresis (IEP) were made using 1% (wt/vol) agarose (Litex, type HSA) in Tris-barbital buffer (29) with 1% (vol/vol) Triton X-100 and adjusted to pH 8.6. The gels were stored at 4°C for at least 2 h before use. Electrophoretic separations were done with a flat-bed electrophoresis unit (Multiphor; LKB, Bromma, Sweden). Gels were connected to a buffer reservoir via cellulose wicks (Ultra-wicks; Bio-Rad, Richmond, Calif.). Plates were maintained at 12°C during electrophoresis by a cooling platform circulator. Electrophoresis was carried out at 6 V/cm (constant) for 60 min as measured on the gels. After electrophoresis, troughs were cut and filled with antisera, and gels were incubated in moist chambers at room temperature or at 4°C.

Agarose gels and buffer solutions used for crossed immunoelectrophoresis (CIE) were as used for IEP above. Antigens were separated in the first dimension as above and were electrophoresed into an adjacent antibody-containing gel in the second dimension at 2 V/cm for 18 h with cooling at 12°C (30). Subsequently, IEP and CIE gels were washed, dried, and stained with Coomassie brilliant blue (30).

EF. A sample of purified *L. donovani* EF from promastigote cultures of strain L-52 (World Health Organization, *Leishmania* Reference Center designation) was generously provided by Gerald Slutzky, Department of Protozoology, The Hebrew University, Hadassah School of Medicine, Jerusalem.

RESULTS

Detection of antigens in used growth media. Rabbit antisera (PCSP-As) directed against purified *L. donovani* PCSP precipitated components present in culture media which had supported promastigote growth (Fig. 1). The specificity of the reaction was revealed by the following controls: normal preimmune rabbit sera did not yield precipitin bands against the used promastigote culture media, nor did the PCSP-As show precipitin lines against unused culture media. A major band, the first to appear in gel diffusions between PCSP-As and used culture media, fused and showed antigenic identity with EF (as provided by G. Slutzky). With additional incubation, other minor precipitin bands became obvious, indicating the presence of several antigens in the used culture media that reacted with PCSP-As.

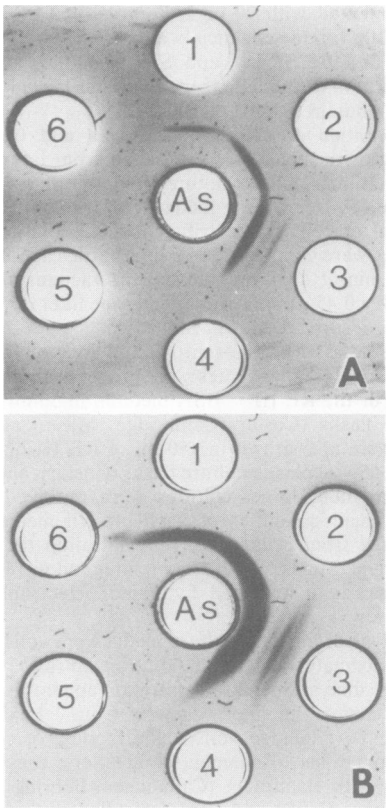

FIG. 1. Antigenic reactivity of PCSP-As with used promastigote culture media and EF. Gel diffusion reactions at (A) 6 h and (B) 27 h between a 3× globulin fraction of rabbit PCSP-As (As) and: two different samples of 100× Amicon PM-10-concentrated, used, day-4 RE III medium (wells 1 and 3); EF, 2.5 mg/ml (well 2); unused 100× concentrated RE III medium control well (4); and preimmune rabbit serum (wells 5 and 6). Several promastigote antigens released in the used culture medium formed precipitin lines with PCSP-As, suggesting their cell surface origin. The heaviest line, which appeared earliest, is contiguous with that of EF, indicating the antigenic identity between EF and the major antigenic constituent in the used RE III medium. Multiple lines between well 3, but not well 1, indicated the presence of several minor antigens in some samples of used media.

In this report, antigens in used growth media that were detected and specifically precipitated in gels with PCSP-As were tentatively referred to as shed membrane antigens (SMA). This provisional designation does not suggest that a mechanism for their genesis has been established.

IEP analyses of antigens present in used growth media. Two major antigens, SMA-A (a faster-migrating antigen) and SMA-B (a slower-migrat-

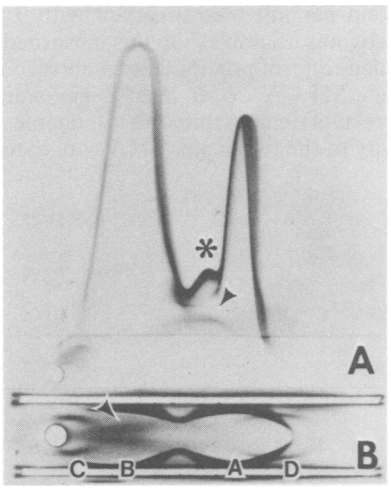

FIG. 2. Resolution of major and minor SMA in Amicon PM-10-concentrated RE III − BSA medium from early-stationary-phase promastigote cultures by (A) CIE and (B) IEP. The anode to the right is the first dimension in both A and B. The major SMA were designated SMA-A and -B. The most cathodic antigen, SMA-C, did not form contiguous precipitin arcs or peaks with SMA-A or -B. The most anodic migrating antigen, SMA-D, formed a precipitin arc that intersected with that of SMA-A. Minor peaks contiguous with that of the major SMA appeared as shoulders (asterisk in A). Other minor antigens (arrowheads) were often detected when high concentrations of antigens were used. Troughs in IEP gels (B) and the antibody gel in CIE analyses (A) contained IgG fractions of PCSP-As. The CIE antibody gel (A) contained 2.4 mg of PCSP-As IgG per ml.

ing antigen), were distinguished in IEP reactions against PCSP-As in all four types of used culture media. Examples of IEP and CIE gel reactions employing used RE III − BSA are shown in Fig. 2. Examples of gel reactions in which the three other used media were analyzed are illustrated below (see Fig. 7A). The immunoprecipitin lines of SMA-A and -B were contiguous, indicating a common antigenic site(s). In some analyses, other components that were contiguous with these major lines were observed in IEP gels. Similarly, in CIE gels these appeared as shoulders of the two major SMA peaks (asterisks in Fig. 2A and 7B). The spur observed on the cathodal edge of SMA-A and anodal edge of SMA-B in Fig. 2A suggested that the PCSP-As contained some antibodies that recognized an antigenic site different from that common to both SMA-A and -B.

Another antigen, SMA-C, which was also present in used culture media, gave weaker precipitin bands in IEP or peaks in CIE gels than did the major SMA-A or -B (Fig. 2). This indicated that SMA-C was either present in lower concentrations than either major SMA in the used medium, or the antisera had lower concentrations of antibodies to this antigen. This precipitin band was not always detected in IEP gels. Antigen SMA-C is apparently antigenically different from SMA-A and -B, as indicated by the lack of antigenic identity among the respective precipitin arcs.

The most anodic antigen detected with PCSP-As, SMA-D, was only occasionally observed in IEP and CIE gels (Fig. 2B). Its appearance depended on the lot of culture medium analyzed, as well as the antiserum used. This precipitin band was not contiguous with, but crossed, the precipitin arc of SMA-A. Other minor antigens were also observed in some analyses (arrowheads in Fig. 2).

Cross-reactivity between antigens of used culture media and the surface of promastigotes. The following studies were carried out to ascertain the relationships between the major SMA and antigens solubilized from isolated PCSP. Preliminary results by gel diffusions (data not shown) demonstrated that used, but not unused, culture media and solubilized PCSP antigens gave lines of antigenic identity against PCSP-As. Also, comparison of electrophoretic mobilities in CIE gels of solubilized PCSP and SMA demonstrated that the major antigens in both of these preparations had similar migration characteristics. The distances from the origin that PCSP antigens migrated corresponded closely to those of SMA-A and -B. These closely corresponding migration distances suggested that the two major PCSP antigens and the two major SMA have similar, although not necessarily identical, properties.

To elucidate the relationship between PCSP antigens and those in used culture media, analyses of SMA and solubilized PCSP were conducted with absorbed antisera. In such assays, samples of PCSP-As previously absorbed with the 10- to 50-kd CHO fraction, which contained SMA-A as shown by IEP (cf. Fig. 7C below), was used. The absorbed PCSP-As did not form precipitin bands against the used culture media in IEP gels corresponding to either SMA-A or -B. Moreover, no precipitin arcs were detected in IEP reactions between the ≥50 kd CHO fraction of used media and the above absorbed serum (Fig. 3B). A concentrated, unfractionated sample of used RE III medium analyzed in the same manner showed neither SMA-A nor -B arcs, but an SMA-C arc was present (Fig. 3C). Thus, SMA-A absorbed antibodies to SMA-A and -B but not antibodies directed against SMA-C, which further verified that the two major SMA had common antigenic sites and that SMA-C was antigenically distinct.

The IEP and CIE patterns of Triton X-100 extracts of *L. donovani* PCSP indicated that the

antiserum contained precipitating antibodies to a variety of antigenic constituents of the PCSP (Fig. 3D and 4A). Approximately 16 components were resolved by CIE. The same Triton extract was also analyzed by both IEP and CIE, employing the PCSP-As preabsorbed with the 10- to 50-kd CHO fraction of used culture medium. In such gels (Fig. 3E and 4B), the major immunoprecipitin peaks were absent, further indicating that SMA-A and -B and the major PCSP antigens are antigenically related.

To further establish the relationship between the antigens in PCSP and the used culture medium, we performed the following experiments. Azide-free PCSP-As IgG fraction was absorbed with live, intact promastigotes and tested for the ability to precipitate used culture medium antigens. A 1-ml amount of the IgG fraction (30 mg of protein per ml) was absorbed with 3×10^9 promastigotes. Samples of this absorbed fraction failed to form precipitin arcs corresponding to either SMA-A or -B in IEP gel reactions. These results demonstrated the antigenic cross-reactivity of the two major SMA with externally

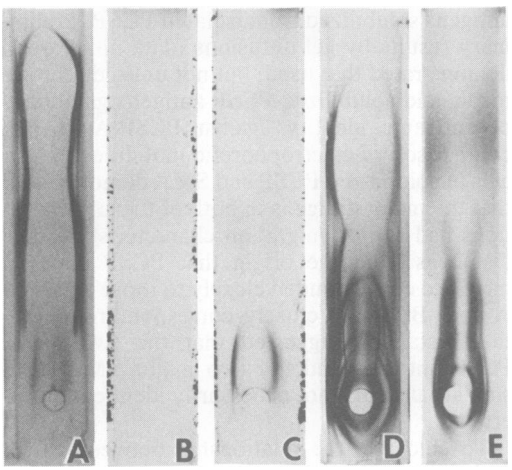

FIG. 3. Absorption of antibodies to SMA-B and several cell surface antigens by SMA-A. A sample of concentrated day-4 RE III medium (A) analyzed by IEP illustrates the typical pattern showing SMA-A, -B, and -C. In this analysis, unabsorbed PCSP-As was placed in the troughs. The globulin fraction of this antiserum was also absorbed with the 10- to 50-kd CHO fraction of used RE III medium (which contained SMA-A as shown in Fig. 3B, C, and E. The ≥50-kd CHO fraction of used RE III medium (which contained SMA-B, as shown by other IEP analyses), was analyzed by IEP, using this absorbed serum (B). The absence of precipitin arcs indicates antigenic cross-reactivity between SMA-A and -B. A sample of unfractionated, concentrated day-4 RE III medium was similarly analyzed with the absorbed serum (C); the presence of precipitin arc SMA-C indicates the lack of antigenic cross-reactivity between SMA-A and -C. A Triton X-100 extract of PCSP was analyzed by IEP, using PCSP-As (D) and the same globulin fraction after absorption with the 10- to 50-kd CHO fraction of used RE III medium (E). Several precipitin arcs present in the IEP gel shown in (D) were absent in the gel shown in (E), indicating that SMA-A cross-reacted with several PCSP antigens.

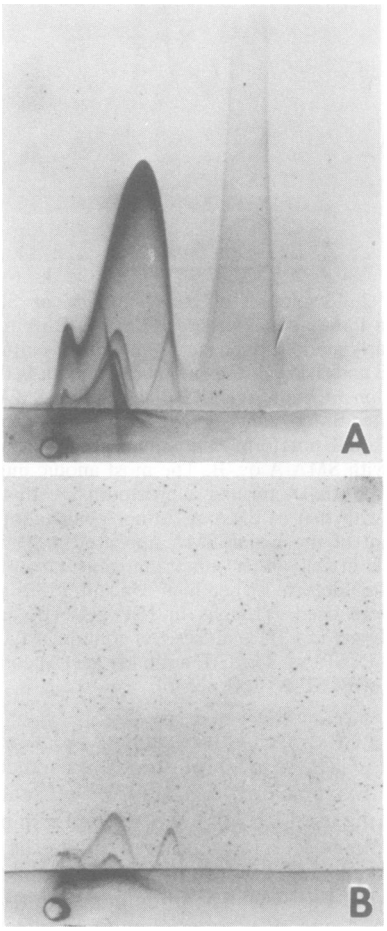

FIG. 4. Absorption of cell surface antigens with SMA-A from the used culture medium, as resolved by CIE. Triton X-100 extracts of PCSP were separated in the first dimension by electrophoresis (anode to the right) and then electrophoresed into PCSP-As-containing gels in the second dimension (anode at the top). The antibody gel shown in Fig. A contained 6% (vol/vol) of the 3× globulin fraction of PCSP-As. This analysis resolved about 16 precipitin components. The antibody gel B contained 4% (vol/vol) of the same 3× PCSP-As globulin fraction as used in gel A, except that it had been preabsorbed with the 10- to 50-kd CHO fraction (which contained SMA-A, as shown by other IEP analyses). The two major precipitin peaks, as well as several other smaller components seen in CIE gel A, were absent in CIE gel B, indicating that SMA-A shared antigenic cross-reactivity with components in the PCSP. The absence of the major antigen peaks is obvious in Fig. 4B.

oriented components in the surface of intact promastigotes. The results of these absorption studies were equivocal with regard to the relationship between the minor used culture medium components SMA-C and -D and the exposed promastigote surface antigens.

The addition of PCSP-As to a suspension of live, intact promastigotes rapidly agglutinated the cells; aggregates of hundreds of cells formed within 15 min. Addition of the crude CHO fraction inhibited the agglutination; only a few aggregates of three to four promastigotes could be detected after 1 h of incubation. Controls with PBS alone, preimmune sera, or PBS with the crude CHO fraction from used culture media did not cause distinct cellular agglutination. The globulin fraction of PCSP-As that was preabsorbed with the 10- to 50-kd CHO fraction from used culture media was also tested for its ability to agglutinate cells. Promastigotes mixed with this preabsorbed globulin fraction did not immediately agglutinate. Aggregates of 6 to 10 cells were observed after 1 h of incubation, although most cells were free and not part of aggregates.

The results strongly suggest that SMA-A and -B of used culture media are antigenically cross-reactive with those antigens exposed on the surface of promastigotes. Further, these components are the major determinants recognized by the rabbit hyperimmune serum to isolated PCSP and responsible for the observed agglutination.

Accumulation of SMA in used growth media. It seemed possible that the accumulation of SMA in culture media might be due to cell lysis. To assess this possibility and to examine SMA production rates in different culture media, we prepared samples from three different used growth media, i.e., 199 + FBS, RE III, and RE III − BSA. Quantities of SMA present in these various media were estimated by rocket IEP (Fig. 5). Maximum peak areas were assumed to be proportional to the concentration of SMA-A or -B or both. Antigens SMA-C and -D could not be specifically identified in these rocket IEP gels; however, minor components were often detected within the rocket peak (arrow in Fig. 5B). Hence, the maximum peak areas of rockets in these analyses do not represent total SMA in culture media. Photocopies of the gels were made, and individual peaks were excised and weighed. The smallest peak obtained in these studies was arbitrarily designated as 1 immunoprecipitin unit (IPU; Fig. 6). Quantitation of SMA represented by other peak weights was calculated as direct proportions to that smallest peak.

Growth curves of *L. donovani* promastigotes in three different culture media are shown in Fig. 6. The doubling time of cells during the log phase of growth was: 199 + FBS, 14 h; RE III, 19 h; and RE III − BSA, 17 h. A distinct stationary phase was observed with organisms grown in 199 + FBS. Promastigotes grown in the chemically defined media, RE III and RE III − BSA, reached the same maximal densities as those in 199 + FBS, but these cultures rapidly entered a death phase. Dead cells were first seen at day 9 in 199 + FBS cultures and at day 6 in RE III and RE III − BSA cultures.

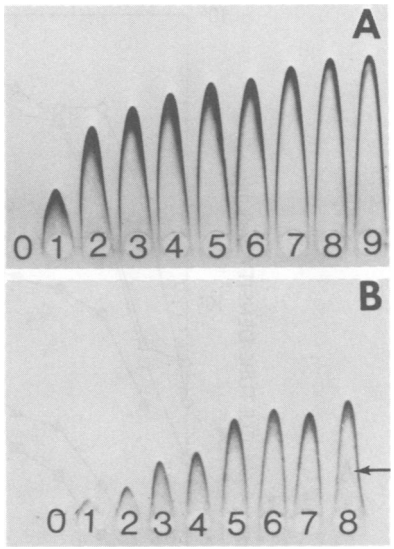

FIG. 5. Quantitation of SMA in used culture media via rocket IEP. Examples of rocket IEP analyses for quantitation of SMA accumulated with culture age are shown in (A) (199 + FBS) and (B) (RE III). The numbers indicate the culture age in days. In these gels, antigen wells were filled with 20 μl of unconcentrated, used media obtained by direct membrane filtration of cultures. Gels shown in this figure contained 4% (vol/vol) of the PCSP-As IgG fraction (anode at the top). When volumes of antigens or antisera concentrations or both in gels were altered to obtain complete, quantifiable peaks, an antigen sample, previously analyzed on a 4% antibody gel, was employed for proper calibration. Minor precipitin peaks were often detected within the largest peak (arrow in B) and were probably due to minor antigens present in used culture media, e.g., SMA-C or -D. These analyses showed that SMA accumulated in media during culture growth and could be quantified and compared with respect to age and other culture conditions.

The amount of SMA produced during promastigote growth in these three different media (estimated by rocket IEP as described above) are also shown in Fig. 6. In 199 + FBS, SMA accumulation was rapid during log phase, then progressed at a constant but lower rate during stationary phase. In RE III and RE III − BSA media, although the death phase was accompanied by a rapid decrease in cell numbers (cell lysis), SMA levels in the media remained con-

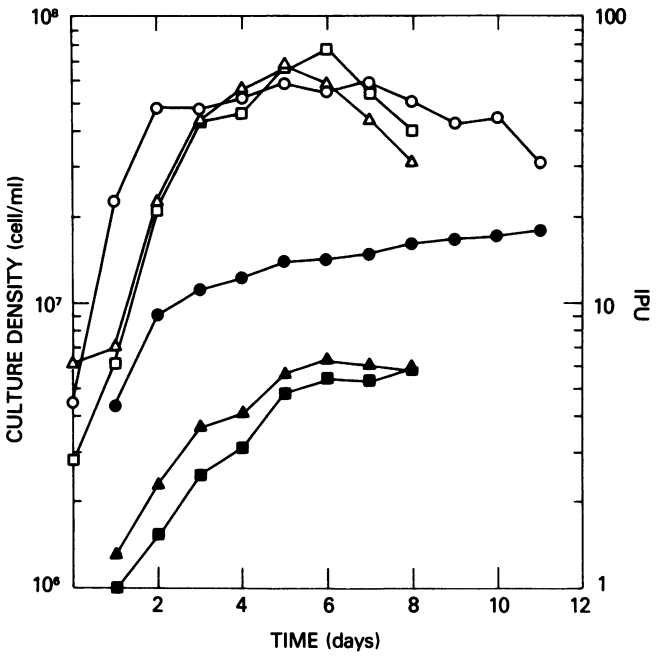

FIG. 6. Comparison of *L. donovani* promastigote growth curves and the corresponding accumulation of SMA in three different culture media. Growth curves of promastigotes in 199 + FBS (○), RE III (△), and RE III − BSA (□) show that maximal cell densities achieved in the three media were similar. However, cells in 199 + FBS showed a distinct stationary phase, whereas those grown in chemically defined media (RE III and RE III − BSA) entered a death phase immediately after reaching maximal cell densities. The accumulation of SMA in 199 + FBS (●), RE III (▲), and RE III − BSA (■) with age was estimated by rocket IEP analyses. The maximum peak area of the smallest peak obtained by rocket IEP in these studies was arbitrarily designated as 1 IPU. Peak areas obtained from the rest of the samples of used culture media were calculated as directly proportional to the area of the smallest peak. Accumulation of SMA was greatest in the serum-supplemented medium, 199 + FBS, and least in the chemically defined medium lacking BSA. These quantitations of SMA also show that, although the cultures were in a death phase during which extensive cell lysis occurred, the amount of SMA in the medium remained constant. This is particularly evident in RE III and RE III − BSA cultures and indicates that significant amounts of SMA were apparently not released from intracellular stores upon cell lysis. The data represent single experiments for 199 + FBS and RE III − BSA and the mean of two experiments for RE III.

stant. These results suggest that there were no detectable soluble stores of intracellular components antigenically related to SMA that were released by the lysis of promastigotes.

The rates of SMA production (IPU/day) during the log phase of growth were: 199 + FBS, 3.46; RE III, 1.21; and RE III − BSA, 0.75. Results of the rocket IEP assays indicated that, on a per cell basis, greater detectable quantities of SMA accumulated in the serum-containing growth medium. The cumulative results of these assays suggested that the major SMA were released continuously into the culture media by intact, live promastigotes throughout their cell cycle, and not produced as a result of their lysis.

Physicochemical characteristics of SMA. To determine the relationship between the two major antigenically cross-reactive components SMA-A and -B, we carried out the following preliminary studies. Antigen SMA-A was readily separated from other SMA components by ultrafiltration through an Amicon XM-50 membrane filter (cf. 10- to 50-kd CHO fraction; Fig. 7C). SMA-B was retained by this filtration procedure, residual SMA-A in the ≥50-kd CHO Amicon XM-50 fraction was subsequently removed from SMA-B by DEAE-cellulose column chromatography (manuscript in preparation; e.g., Fig. 7D).

Analysis of *L. donovani* promastigote EF (15, 24–26) indicated that its migration in IEP gels was similar to that of SMA-A (Fig. 7E). Current gel diffusion results, as indicated above, demonstrated antigenic identity between this EF and SMA-A. Furthermore, PCSP-As preabsorbed with the 10- to 50-kd CHO fraction did not form precipitin lines against EF in gel diffusions (data not shown).

The following observations indicated that SMA-A and -B, including their antigenic sites, were carbohydrate in nature: (i) immunoprecipitable activity was retained after heating at 100°C

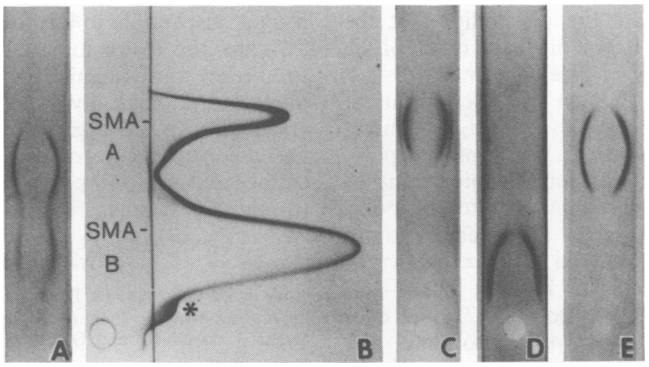

FIG. 7. Separation of SMA-A and -B. Resolution of SMA-A and -B via IEP against PCSP-As (A, unconcentrated Schneider + FBS) and by CIE (B, concentrated RE III). Precipitin reactions between the 10 to 50 kd CHO fraction = SMA-A (C); the chromatographically purified ≥50-kd CHO fraction = SMA-B (D); and EF (E). The asterisk in (B), like the one in Fig. 2A, indicates that minor peaks contiguous with that of SMA-A and -B are often detected at more than one position. Precipitin bands between SMA and PCSP-As were often split in two (C). The appearance of split bands was not a function of refilling of antibody troughs, but appeared to be related to the concentrations of antigens and antibody solutions used. Since polyvalent IgG or globulin fractions were used in these studies, the split was probably the result of different concentrations of IgG molecules that recognized different sites on the antigens.

for 1 h, as well as after lyophilization and repeated freezing and thawing; (ii) antigenic activity remained present in the soluble fraction of 10 or 33% TCA-treated samples; (iii) precipitin activity was recovered in the aqueous phase after extraction with 44% phenol-water; (iv) antigenic activity was unaffected by pronase treatment; (v) precipitin activity was lost after treatment with periodate; and (vi) precipitin activity was recovered from a pellet from *Ricinus communis*$_{120}$ lectin precipitation and its subsequent solubilization with D-galactose.

DISCUSSION

Identity of SMA with externally oriented surface antigens. The present study utilized polyvalent antisera from rabbits immunized with purified PCSP of *L. donovani* to establish a relationship between cellular antigens of these protozoan parasites and antigens operationally characterized as extracellular. Precipitable antigens, designated here as SMA, were found in cell-free supernatant and micro-filtrates of promastigotes cultivated axenically in both serum-supplemented tissue culture media and in chemically defined, macromolecule-free medium. The experimental evidence established an identity, by gel immunodiffusion, between extracellular antigens and antigens of the isolated PCSP. PCSP-As, absorbed with a major extracellular antigen, SMA-A, failed to recognize the other major extracellular antigen, SMA-B, and, most significantly, failed to recognize two major antigens present in Triton X-100 extracts of the PCSP. These two major cell surface antigens corresponded in position to SMA-A, and -B in CIE analyses.

The ability of whole promastigotes to absorb antibodies to SMA-A and -B, as well as the ability of SMA to inhibit promastigote agglutination mediated by PCSP-As, demonstrated the antigenic cross-reactivity between SMA and the major externally oriented cell surface antigens. However, these studies cannot establish whether such antigens are also exposed on the cytoplasmic side of the surface membrane. These promastigote antigens may be exposed on both sides of the membrane in a distribution pattern similar to that suggested for the major plasma membrane lipophosphonoglycan of *Acanthamoeba castellanii* (1, 4).

The identity of the SMA with the cell surface antigens of promastigotes does not presume a mechanism for their origin. The time course studies indicated that the antigens, at least the major antigens, SMA-A and -B, were released during the log and stationary phases of the growth cycle and that they did not arise in the media primarily as a result of cell lysis or damage. Further studies are required to determine whether SMA are released by a shedding or turnover of membrane components. Such studies will require an understanding of the chemical nature and localization within the cell of the major PCSP antigens which correspond to SMA-A and -B in the medium. The method of preparation of PCSP suggested that these antigens are integral constituents of the membrane. Alternatively, the results do not exclude the possibility that such antigens also exist as peripheral membrane components. Our studies

also did not eliminate the possibility that the presence of SMA in the media is the result of the secretion of SMA by intracellular sites.

Relationship between SMA and EF. In contrast to this study, previous investigation of leishmanial extracellular antigens have employed polyvalent antisera directed against whole promastigotes or crude cellular homogenates or both. The precipitating antigens identified by these reagents have been referred to, by other workers, as excretory factor or EF (15, 20, 24–26), antigenically active glycoproteins (7), and exometabolites (21, 22). However, to date, the cellular origin and localization of these antigens have remained undefined. Currently, it is unclear whether the substances described by these different investigators are, in fact, identical. In this report, we have shown an antigenic identity between EF and the major SMA. Furthermore, we observed that EF and SMA-A had similar electrophoretic characteristics in IEP gels.

Physicochemical characteristics of SMA. Results of various treatments of the extracellular antigens indicated that both SMA-A and -B are CHOs. These results confirmed earlier data for the CHO nature of EF (15, 24, 27). Previously, two forms of EF were reported, and it was concluded that the two forms were the result of adsorption to serum proteins (27). In that regard, we have demonstrated that the distinction between SMA-A and -B in our studies cannot be the result of interactions with serum proteins, as identical results were obtained in the chemically defined medium that lacks proteins (RE III − BSA). Similarly, different forms of leishmanial exometabolites have been identified and attributed to aggregation/degradation processes occurring during their purification and analyses (22). However, preliminary results of metabolic labeling studies lead us to conclude that the presence of SMA-A and -B in our immunochemical analyses cannot be explained by aggregation of chemically identical molecules. Samples of SMA that were metabolically labeled with [^{14}C]inositol or [^{14}C]stearic acid were analyzed by IEP and subsequently subjected to radioautography. The results suggested that SMA-B, but not SMA-A, contained inositol and stearic acid (manuscript in preparation). Purification of the various SMA components is needed to further establish the chemical identity of these molecules.

Significance to SMA. The large amounts of SMA released by promastigotes suggest that these components may be continuously shed by the cells during natural infections of vertebrate or insect hosts. In that regard, EF has been shown to be produced by amastigotes within macrophages (24), and in vitro-generated EF has been used to render normally resistant macrophages susceptible to leishmanial infection (18). To date, the major extracellular antigens have been used to distinguish strains and species of these morphologically indistinguishable protozoans (20). In this regard, further knowledge of the chemical nature of these extracellular and surface antigens will prove useful to better identify these parasites.

ACKNOWLEDGMENTS

We thank T. Nash and J. Dvorak for useful suggestions and for making available their research facilities; C. Grady for technical assistance; and G. Slutzky for the sample of *L. donovani* EF.

This work was supported in part by U.S. Public Health Service grant AI-16530 from the National Institute of Health to M.G.

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