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Exometabolites of *Leishmania donovani* Promastigotes. I. Isolation and Initial Characterization (40293)[1]

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Isolation and characterization of parasitic protozoan exometabolites is of importance because of the possible role these may play in the host-parasite relationship. Since the products of intracellular parasites do become mixed and contaminated with host cell substances, isolation, purification, and characterization of these parasite products becomes difficult.

Leishmania provides an ideal system where the influence of exometabolites produced by an obligative intracellular protozoan parasite upon the host may readily be studied. Leishmanial organisms have two morphological forms: the amastigote, an obligative intracellular form infecting vertebrates, and the promastigote, which exists extracellularly in the insect vector and will grow readily in culture. Both forms have been reported to produce and release exometabolites which demonstrate antigenic identity (1, 2).

Reports to date dealing with substances that accumulate in the media in which promastigotes are metabolizing (metabolized medium) have involved either undefined complex media containing blood proteins (1, 3) or salt solutions (4, 5). In order to determine if promastigote substances were present in metabolized Senekji's medium after promastigote growth, Clinton *et al.* (3) utilized immunoelectrophoretic procedures, reacting metabolized medium against antiserum raised in rabbits to the homologous promastigotes. One band formed between the antiserum and a substance from the metabolized medium. No reaction was observed when nonmetabolized medium was tested. Schnur *et al.* (1) utilized metabolized Feinberg and Whittington's medium and reacted this with rabbit antipromastigote hyperimmune serum and demonstrated multiple bands (termed EF) by diffusion in gels. Since Schnur *et al.* obtained their metabolized medium from cultures of promastigotes in log phase, they concluded that the EF substances were exometabolites and not products of lysis. In addition, the molecular weight of the EF substances was within the range of 25,000 and 70,000, but they were not immunogenic when injected into rabbits. Decker and Honigberg (6), however, reported successful induction of antibodies in mice to the exometabolite. Results utilizing less defined media suggest that promastigotes of *Leishmania* produce exometabolites, but there is no agreement as to their number and immunogenicity (1, 3, 6). The lack of agreement in the data may be attributed to the different media used to culture the promastigotes.

Media used in *in vitro* culture should be defined and protein free to facilitate recovery of exometabolites more closely resembling the native form released from the parasite. Greenblatt and Glaser (4) used Locke's solution with glucose at 37° to maintain promastigotes and found a variety of molecules including various amino acids, hypoxanthine, guanosine, uracil, and ribose in the medium. They did not detect any large molecules and concluded that the low molecular weight substances found in the metabolized medium resulted from leakage and not gross lysis. On the other hand, Decker and Janovy (5) in a similar study detected not only small molecules but also proteins and RNA. Thus, while salt solutions may be ideal for recovery of leakage products from promastigotes, they may not adequately support complete metabolism of the organisms. Measurable quantities of larger molecular weight excretion–

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secretion metabolic products may not accumulate. On the other hand, the higher molecular weight products detected could be the result of lysis.

More recently Slutzky and Greenblatt (7) isolated a substance by degradative isolation techniques (boiling and 33% trichloroacetic acid solution) from proteid *L. tropica* metabolized medium. The substance isolated was initially associated with medium protein, was immunologically active and carbohydrate rich. The isolated entity did not pass through a 30,000 mol wt exclusion membrane. Little or no protein was reported to be associated with the isolated entity.

The object of this study was to isolate and characterize the metabolic by-products of *L. donovani* promastigotes in their native form. To accomplish this log phase promastigotes of *L. donovani* were maintained in protein free tissue culture medium to minimize the interference of lytic by-products. The metabolized medium was then fractionated and examined spectrophotometrically and serologically.

Materials and methods. Amastigotes utilized to initiate promastigote cultures were obtained from the spleens of hamsters infected with the 3S strain of *L. donovani* (8). Spleens were homogenized in sterile phosphate-buffered (pH 7.0) physiological saline and amastigotes isolated by differential centrifugation (9). All cultures were initiated at a density of 5×10^4 organisms per ml and subcultures were made when the density of a culture reached 2×10^7 promastigotes per ml. Promastigotes utilized to generate metabolized culture medium were never less than 4 nor more than 15 subcultures removed from the initial amastigote-seeded culture. All cultures were incubated with an atmosphere of 5% CO_2 in air at 25 ± 0.1°.

The culture medium utilized to grow promastigotes (growth medium) consisted of 9 parts Medium 199 with Hanks' salts (Gibco) and 1 part whole defibrinated rabbit blood (Pel Freeze). The blood was centrifuged (4 hr at 2000*g*) before inclusion into the medium to separate serum from cells. Serum was inactivated at 56° for 30 min and stored at −20° until used. Cells were washed in excess Hanks' balanced salt solution (Gibco) 5 times and lysed in a volume of double distilled water equal to 10× the packed cell volume. Cell ghosts were removed by centrifugation (24 hr at 200*g*) and the supernatant utilized in the medium. To prepare 1 liter of growth medium, 100 ml of Medium 100 (10×) was added slowly to 500 ml of lysate and a sufficient amount of double distilled water added to bring the volume to 950 ml. The pH was maintained at 7.2 by addition of $NaHCO_3$ as needed. Serum (50 ml) was then added and the medium sterilized by filtration through a 0.22 μMillipore filter.

Medium used to maintain promastigotes (maintenance medium) consisted of Medium 199 with Hanks' salts (Gibco) and 25 m*M* Hepes buffer (Sigma). The pH was adjusted to 7.2 with 1 *N* HCl or NaOH. The maintenance medium was sterilized as described above.

Promastigotes were allowed to metabolize both growth and maintenance media. Growth medium cultures were initiated at a density of 5×10^4 organisms per ml. When the promastigote density reached 8×10^6 per ml (mid log phase), the cultures were centrifuged (20 min at 2000*g*) separating promastigotes from the medium. Organisms were washed 3 times in excess Hanks' balanced salt solution and resuspended in maintenance medium at a density of 10^7 promastigotes per ml. Cultures in maintenance medium were incubated 8 hr at 25° with a 5% CO_2 in air atmosphere. Promastigotes were removed by centrifugation (1 hr at 2000*g*) and the metabolized medium was filtered through a 0.22 μMillipore filter, concentrated 10× by lyophilization and stored at −20°.

Two ml aliquots of 10× concentrated metabolized maintenance medium were fractionated on a column (1.6 × 80 cm) of super fine grade Sephadex G25 (Pharmacia). Void volume was 54 ml, bed volume was 160 ml and flow rate was 7 ml per hr. The eluant used was a 5% acetic acid solution in distilled water. The column was characterized utilizing α-melanocyte stimulating hormone, mol wt 1910; Bradykinin, mol wt 1204; and gastrin pentapeptide, mol wt 768, all purchased from Calbiochem. Elution values were 94, 120, and 145 ml respectively. Each standard was applied to the column as a 1 ml vol containing 50 μg peptide. Elution volume was determined from the maximum of the elution

peak. Elution values for the standards graphed against the log of their molecular weights approximate a straight line.

Two ml fractions were collected from the column and analyzed on a Beckman DB Model 24 spectrophotometer. Absorption spectra were obtained between 190 and 350 nm.

The amount of peptide present in a fraction was estimated photometrically by the method of Mayer and Miller (10). Fractions were lyophilized to dryness and redissolved in solvent (0.15 *M* NaF in glass double distilled water). The blank contained solvent only. Absorbance was measured at 193 nm and a standard curve generated using bovine serum albumin, α-melanocyte stimulating hormone, gastrin pentapeptide and Bradykinin (Calbiochem). The standard curve developed here was indistinguishable from that presented by Mayer and Miller with 11 μg/ml protein yielding an absorbance value of 0.7. Direct proportionality between concentration and absorbance was applicable for all standards up to an absorbance of 0.7. Since all fractions in which protein amount was estimated had absorbance values greater than 0.7, aliquots of fractions were diluted with solvent until an absorbance value of 0.7 was attained. The amount of protein in a fraction was calculated by multiplying the dilution factor $\times$ 11 μg/ml.

The amount of sugar present in fractions was determined by the procedure of Dubois *et al.* (11). A standard curve for D-galactose was generated which was indistinguishable from that presented by Dubois *et al.* The average value for triplicate samples containing 10 μg of D-galactose was 0.11 absorbance units at 490 nm.

Specific chemical tests for tryptophan and tyrosine were performed on selected fractions by the procedure of Fischl (12) and the method of Udenfriend and Cooper (13) as modified by Massin and Lindenberg (14) respectively. Controls were composed of 50 μg/ml solutions of tryptophan, tyrosine and cysteine.

Anti *L. donovani* promastigote immune serum was raised in rabbits by injecting a homogenate composed of freeze-thawed promastigotes in saline and FCA (1:1). Each rabbit received a total volume of 1 ml, containing 21 mg *N* (determined by Kjeldahl procedure [Campbell *et al.*, 15]) delivered in 0.1 ml aliquots at one time to 8 sc sites on the back and 2 im sites in the hind legs. The animals were bled 30 days after immunization. Serum was recovered by centrifugation (1 hr at 2000*g*) and stored at $-20°$.

Test antigens were prepared by mixing (6:1) metabolized maintenance medium (free of serum) with nonmetabolized growth medium (containing serum) and concentrating tenfold by lyophilization. Control antigens were nonmetabolized growth medium and nonmetabolized maintenance medium prepared in the same manner.

The microsolutes in each sample were exchanged by diafiltration (16) and standardized using a 500 mol wt cutoff ultrafiltration membrane (UM 05) with a Model 12 stirred cell (Amicon). Five sample volumes of barbital buffer (17) were exchanged with a predicted 99+% complete exchange of microsolute (16).

Gel diffusion plates were prepared by pouring 10 ml melted agar solution (1% Difco Bacto Agar in barbital buffer [17] with 0.1% sodium azide) into a 9 cm-diameter petri dish. Wells (5 mm O.D.) were cut in the agar 7.5 mm apart (center to center). After the wells were filled with either antiserum or antigen solution, the plates were incubated 48 hr in a humid atmosphere at 25°. Precipitin lines appeared within 1 to 2 days but were allowed to develop for a total of 4 to 7 days. Gels were washed free of nonreacting protein with barbital buffer (17) for 48 hr (4 changes of buffer) and stained wet with a saturated solution of picric acid in 1% acetic acid.

Results. When promastigote metabolized and nonmetabolized growth media were tested against rabbit antipromastigote immune serum by gel diffusion, the metabolized medium reacted forming multiple precipitate bands. This confirmed earlier reports that exometabolites were present in the metabolized growth medium and would react with specific antiserum (1, 3). When promastigote metabolized maintenance medium was tested against the same antiserum, no reaction occurred. This suggested that the presence of serum protein was necessary for the exometabolite to react with antibody.

To determine whether serum protein was indeed essential for formation of specific pre-

cipitates, metabolized and nonmetabolized maintenance media were mixed with nonmetabolized growth medium, the microsolute environment standardized, and reacted with immune serum. The mixture containing metabolized maintenance medium yielded multiple precipitate bands identical to the ones observed when metabolized growth medium was used as the reacting antigen (Fig. 1). No reaction occurred with the nonmetabolized medium.

When metabolized maintenance medium was fractionated, spectrophotometric analysis at 274 nm revealed two major fractions (A and B) (Fig. 2) with elution values of 101 and 122 ml respectively. Ultraviolet absorption spectra of these major fractions from 190 to 350 nm are shown in Fig. 3. None of the fractions from nonmetabolized maintenance medium demonstrated either of the major peaks shown in Fig. 2.

When all fractions collected after column chromatography of either metabolized maintenance medium or nonmetabolized maintenance medium were mixed with nonmetabolized growth medium and tested against antipromastigote immune serum, only Fraction B reacted to form precipitate bands (Fig. 1). These precipitate bands demonstrated reactions of identity with those formed against antipromastigote immune serum using promastigote metabolized growth medium as the reacting antigen. When Fraction A (10 absorbance units) was mixed with the antiserum prior to reaction with Fraction B, no evidence of neutralization was observed.

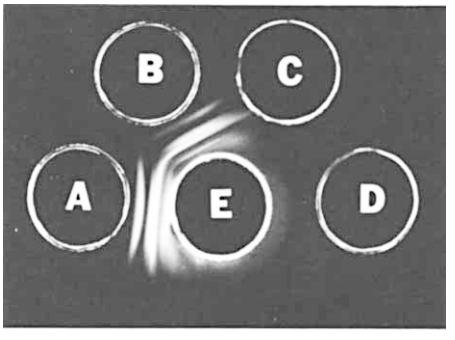

FIG. 1. Gel diffusion plate depicting reactions of promastigote metabolized growth medium (well A), concentrated promastigote metabolized maintenance medium mixed with nonmetabolized growth medium (well B), concentrated promastigote metabolized maintenance medium (well C) and concentrated nonmetabolized maintenance medium mixed with nonmetabolized growth medium (well D) against rabbit antipromastigote immune serum (well E).

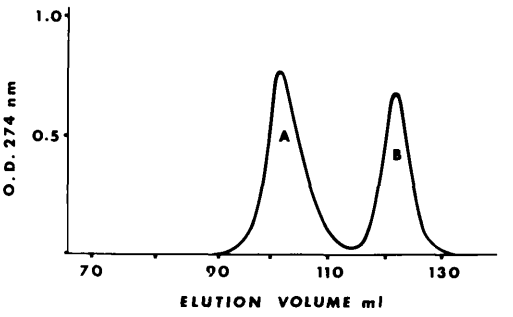

FIG. 2. Sephadex G25 gel filtration profile of metabolized maintenance medium at 274 nm.

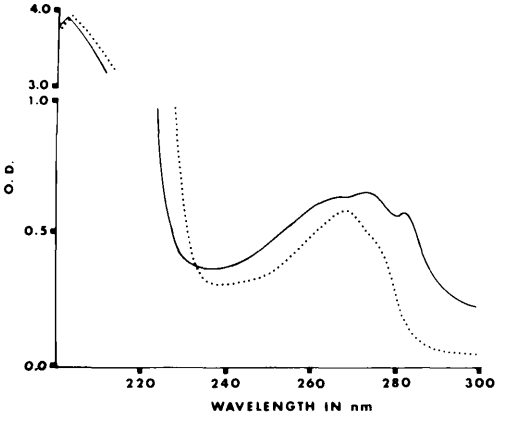

FIG. 3. Ultraviolet absorption spectra of gel filtration Fraction A (-----) and Fraction B (——), pH 7.2.

Fraction A and B samples with absorption values of 3.1 (at 293 nm) were estimated to contain approximately 48 μg peptide and 10 μg sugar per ml. Fractions from the column which eluted both immediately before and after Fractions A and B were determined not to contain sugar.

Discussion. The results suggest that at least two low molecular weight substances are recoverable from promastigote metabolized protein free medium during the log phase growth of the organisms. No high molecular weight substances were detected as might have been anticipated if the recovered substances were the result of promastigote lysis. Microscopic examination of log phase cultures revealed no lysed organisms suggesting that recovered substances are indeed exometabolites and not products of autolysis. Spec-

tral data shown in Fig. 3 suggest the presence of peptide bonds (10) with tyrosine present in Fraction A and tryptophan in Fraction B (18). The presence of these amino acid residues was confirmed by colorimetric procedures. Detection of sugar in Fractions A and B suggests that the substances may be glycopeptides. Since the molecules appear to be of low molecular weight and the ratio of protein to sugar is approximately 5:1, the carbohydrate entity is most likely composed of only a few units.

The molecular weights of the substances in Fractions A and B appear to be in the range of 800–1900 in that their elution values were intermediate between those of gastrin pentapeptide (mol wt 768) and α-melanocyte stimulating hormone (mol wt 1911) (see Andrews [19]). It is premature at this time to assign a more precise molecular weight. The estimated molecular weight of recovered substances suggests glycopeptides composed of from 5 to 13 amino acid residues. Peptides of this size would be expected to act as simple haptens (20).

Generally low molecular weight substances do not induce an imune response unless conjugated to a larger carrier molecule (21). The conjugation of low molecular weight material to a protein carrier endows that conjugated antigen with multivalency with respect to the haptenic moiety (20). The exometabolites appear to act as monovalent haptenic groups. The data suggest that the simple substance isolated in Fraction B attaches to sites on the protein molecule making the conjugated molecule multivalent with respect to that site and thus able to form precipitates when reacted with antipromastigote immune serum. This interpretation is supported by the fact that the bands formed with the promastigote metabolized growth medium are identical to those observed when the substance in Fraction B is mixed with protein. The substance in Fraction A did not form precipitates when tested against antipromastigote immune serum. This may have occurred because no polyvalent entities formed or because there was insufficient antibody present specific for this moiety.

The exometabolite produced by *L. tropica* has been reported to be a carbohydrate-rich substance that does not pass through a 30,000 mol wt exclusion membrane (7); however, it has been demonstrated to be adsorbed initially to medium proteins. While it is not impossible that *L. donovani* and *L. tropica* produce physically distinct exometabolites, the major differences reported may result from the method of isolation. The *L. donovani* exometabolite reported here was isolated by gentle procedures under mild conditions while Slutzky and Greenblatt utilized more harsh procedures.

Fraction B exometabolite is released by both amastigotes and promastigotes as evidenced by the fact that reactions of identity occur when promastigote metabolized growth medium and amastigote infected spleen homogenate supernatant react with antipromastigote immune serum (2). *Leishmania donovani* promastigote metabolized growth medium has been used as a vaccine and induced specific protection against amastigote challenge (22). If the protective substance in metabolized medium is a conjugated antigen, then Fraction B exometabolite may be the antigenic determinant responsible for the protection. Work is proceeding to determine if Fraction B, after conjugation to a protein carrier, will act as an immunogen and induce specific protection.

Summary. Two exometabolites have been demonstrated to accumulate in protein free culture medium in which log phase promastigotes of *L. donovani* are metabolizing. These molecules demonstrate gel filtration characteristics suggesting a molecular weight in the range of 800–1900. The ultraviolet absorption spectra of the exometabolites suggest the presence of peptide bonds with tyrosine present in one and tryptophan in the other. Sugar was demonstrated to be associated with both Fractions A and B, suggesting the exometabolites are glycopeptides. The exometabolite in Fraction B did not react with specific antibody to form precipitates unless it was in combination with serum protein. The data strongly suggest that the exometabolite conjugates with protein forming a multivalent entity.

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