ISOLATION OF A CARBOHYDRATE-RICH IMMUNOLOGICALLY ACTIVE FACTOR FROM CULTURES OF *LEISHMANIA TROPICA*

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1. Introduction

Leishmania promastigotes growing in culture have been shown to excrete a carbohydrate-rich, immunologically active substance that was species specific [1]. Recently preparations of such an excreted factor (EF) from *L. donovani* were enriched by precipitation with ammonium sulfate and the EF was found to be a polysaccharide containing few, if any, amino acids [2]. Extraction of growth medium with acetone has also been used to concentrate a soluble antigen of *L. tropica* [3].

In the current report we describe a simple, reproducible procedure for the isolation of EF from culture medium in which *L. tropica* had been grown. We show by biochemical and immunological procedures that EF apparently contains two components, a carbohydrate segment produced by the parasite and a protein segment incorporated from the growth medium.

2. Materials and methods

L. tropica (LRCL 137) was grown in Panmede medium [1] supplemented with 10% rabbit serum. Organisms were maintained by weekly transfers on biphasic NNN medium, but were transferred after washing to the monophasic medium. Anti-sera to *Leishmania* were prepared by six i. v. injections of living promastigotes into rabbits. The anti-rabbit serum was a commercial preparation (Miles-Yeda, Rehovot, Israel). Rabbit serum albumin was prepared by the method of Michael [4].

Immunological activity was determined by either a simple slide precipitation test or by double gel diffusion on agar plates. Immunoelectrophoresis procedures were employed in attempts to identify the carrier protein. Polyacrylamide gel electrophoresis was carried out on gels containing 5.6% acrylamide and 0.1% SDS. Gels were prepared as described by Fairbanks et al. [5] with modifications described elsewhere [6]. The amounts of sugar and protein in samples were determined by the procedure of Dubois et al. [7] and Lowry et al. [8] respectively.

Specific sugars were determined by gas–liquid chromatography [9] and by the sialic acid assay of Warren [10]. Lectin activity was assayed by the ability of EF to inhibit lectin-mediated agglutination of erythrocytes. Lectins were kindly provided by Dr Itzhak Kahane. Amino acids were determined after hydrolysis in 6 N HCl for 20 h at 100°C in a Beckman model 120 amino acid analyzer.

3. Results and discussion

Organisms were removed from the medium by centrifugation at low speed and the supernatant fluid

was passed through a Seitz filter. The medium was then dialyzed against frequent changes of distilled water for 48 h (fig.1). Double gel diffusion tests for the leishmanial antigen in concentrated dialysate failed to detect any antigenic materials small enough to pass through the dialysis membrane. After removal of sediment by centrifugation, the medium was boiled for 30 min and the resulting precipitate removed by further centrifugation. The cleared medium was freeze-dried and then redissolved in distilled water to one-twentieth of the original volume. This material was made up in 10 X vol. methanol and the addition of approx. 0.25 ml 30% sodium acetate to 11 ml of this sample produced a heavy, cloudy precipitate. Control medium treated in the same fashion yielded a barely visible precipitate containing about 6% as much protein, as determined by comparison of optical densities at 280 nm.

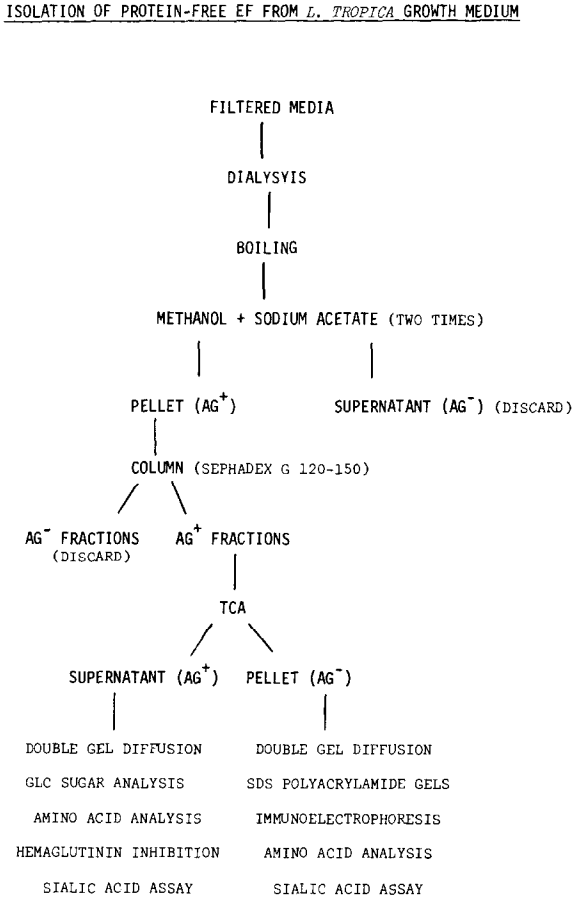

Fig.1. Isolation of protein-free EF from *L. tropica* growth medium.

Material from the pellet of infected medium precipitated with both anti-rabbit serum and anti-*L. tropica* serum and was water soluble. This material was applied to a column of Sephadex G-120–150 and was eluted with distilled water. Typically, the first two fractions immediately following the void volume contained considerable amounts of protein but no *Leishmania* antigens. The next several fractions, of decreasing protein content, reacted with anti-*L. tropica* serum. These fractions were pooled, frozen and lyophilized. This material, composed of about 80% protein and 20% carbohydrate, precipitated with both anti-rabbit and anti-*L. tropica* sera.

The dried protein–carbohydrate complex was dissolved in distilled water and the carbohydrate moiety was stripped from the protein by the addition of trichloroacetic acid (TCA) to a final concentration of 33% (fig.2). The protein pellet was dialyzed extensively to remove TCA and was found to be free of sugars and to precipitate with anti-rabbit serum but not with anti-*L. tropica* serum. The supernatant was extracted 4 times with water saturated ether and the pH adjusted to 7.0. This fraction contained the purified EF–carbohydrate component, which precipitated with anti-*L. tropica* serum but not with anti-rabbit serum (fig.3).

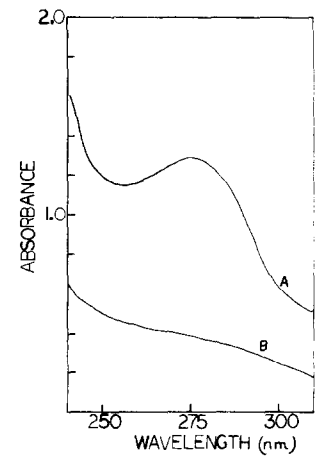

Fig.2. Ultraviolet spectrum of EF-solution before and after extraction with TCA. Absorption was monitored on a Unicam Spectrophotometer.

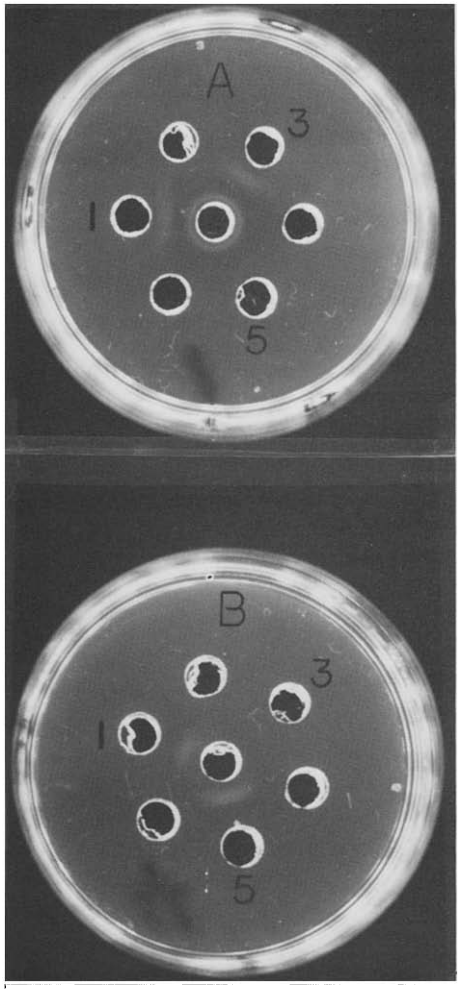

Fig.3. Double gel diffusion plates showing immunological reactivities of the EF preparation before TCA extraction (wells numbered 1) and of the separated protein (wells numbered 3) and carbohydrate (wells numbered 5) components when tested against anti-rabbit serum (center well, Plate A) and anti-*L. tropica* serum (center well, plate B).

The isolated carbohydrate moiety could not be located on polyacrylamide gels stained with Coomassie Blue or the periodate–Schiff reagent, suggesting that it contains little or no protein or sialic acid [11], a finding confirmed by the inability of the Warren sialic acid assay to detect any of this sugar. The carbohydrate moiety did not pass through Amicon P10 (10 000 mol. wt exclusion) or P30 (30 000 mol. wt exclusion) membranes, but any attempt to assign a molecular weight would be premature. Analysis of the carbohydrate by gas–liquid chromatography yielded 45.6% glucose, 39.9% galactose, 14.2% mannose and a trace of glucosamine. Since the material inhibited hemagglutination by concanavalin A only poorly, and did not inhibit hemagglutination by wheat germ agglutinin, soy bean agglutinin, *Ricin communis* agglutinin, peanut agglutinin or abrin, it may be that the ends of the molecule are masked by amino acids. However, amino acid analysis failed to confirm the presence of amino acids in the EF–carbohydrate component.

Figure 4 shows a schematic diagram of SDS–polyacrylamide gels of the EF–protein complex during the various stages of isolation. Before TCA treatment the pooled antigenic fractions yielded a single band (gel 3), but after removal of the carbohydrate segment by trichloroacetic acid (TCA), two protein components were found (gel 4), one of which co-electrophoresed with rabbit serum albumin (gel 5).

The intact EF–protein complex moved in immunoelectrophoresis slightly behind albumin, and the post-TCA protein segment co-electrophoresed with albumin. However, perhaps due to the effects

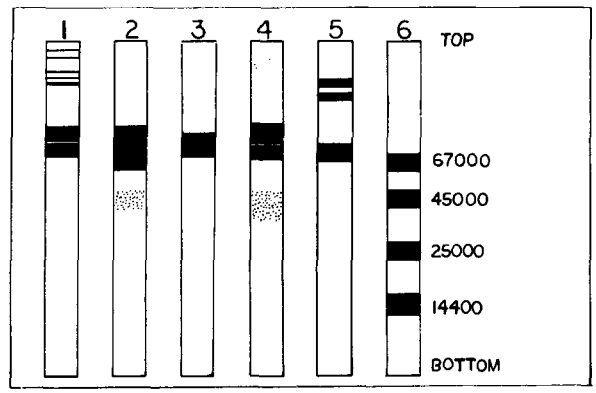

Fig.4. Schematic diagram of SDS–polyacrylamide gel patterns from Coomassie Blue stained gels containing material from various stages of the EF preparation procedure. Gel 1, pre-methanol extraction; gel 2, post-methanol extraction; gel 3, pooled Ag^+ fractions after Sephadex column chromatography; gel 4, post-TCA EF–protein segment; gel 5, rabbit serum albumin prepared by TCA precipitation; gel 6, molecular weight markers (bovine serum albumin, mol. wt 67 000; ovalbumin, mol. wt 45 000; chymotrypsinogen A, mol. wt 25 000; and lysozyme, mol. wt 14 400).

of TCA treatment, the immunoelectrophoretic band of the albumin did not appear as the typical boat shaped curve. As the post-TCA protein yielded two bands on the polyacrylamide gels, it is not possible to conclude that serum albumin exclusively serves as the carrier molecule. Such a role for serum albumin is, however, not unexpected as many types of molecules including fatty acids, sterols, and peptides are known to be transported by serum albumin [12].

The EF–protein complex did not seem to be an artifact of the methanol precipitation procedure. Prior to methanol extraction no reactivity towards anti-*Leishmania* serum could be found that was not also reactive with anti-rabbit serum. When promastigotes were grown in medium containing [^{3}H]glucosamine or [^{3}H]leucine (but not when the medium contained [^{3}H]thymidine or [^{3}H]uridine), the medium was found to contain a radioactively labeled component that could be specifically precipitated with anti-*Leishmania* serum. When this material was electrophoresed on SDS–polyacrylamide gels, a labeled band was found at the same location as that seen for the EF–protein complex on Coomassie Blue stained gels. Furthermore, SDS–polyacrylamide gels of the organisms themselves labeled with [^{3}H]glucosamine or [^{3}H]leucine also contained labeled bands at the same location.

Immunologically active polysaccharides and glycoproteins have wide distribution among the hemoflagellates [1,13] and our methanol extraction procedure may have general application for their isolation. Dwyer et al. have suggested that carbohydrate moieties on the cell surface may facilitate binding of the parasite to the alimentary tract of its insect host [14]. Also related to the flagellate form of the organism is the finding that conditioned medium (pre-enriched with EF) abolishes the lag phase of promastigote growth [15]. EF may also have a function in amistagote development. We have found that macrophages grown in the presence of EF show changes in the distribution of incorporated [^{3}H]-glucosamine suggestive of those found in macrophages infected with *L. tropica*. Interaction of EF with macrophages may be involved in the ability of EF to permit *Leishmania* to infect otherwise resistant macrophages [16].

An intriguing question is why the EF–carbohydrate is bound to a host carrier protein. A possible explanation may involve the masking of EF by host antigens in order to prevent the detection and removal of EF from the host environment. Such a 'camouflage' mechanism could serve to maintain a concentration of EF in the host necessary to condition the host's macrophages for infection.

Acknowledgement

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