ANTIGENICITY OF CELLULAR AND SUBCELLULAR FRACTIONS OF *LEISHMANIA DONOVANI*

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ABSTRACT

The antigenic components of *Leishmania donovani* promastigotes were separated by centrifugation and sonic disruption. The DNA, protein and carbohydrate contents of the soluble, particulate and subcellular fractions were estimated. The molecular weights of the various components were determined by SDS-PAGE. These results showed that the various antigenic components of *L. donovani* promastigotes contain both protein and carbohydrate moieties. Antigenic proteins with molecular weight in the region of 26,000 (whole antigen) and 50,880 (soluble fraction), 65,000 (subcellular fraction I), 53,000 (subcellular fraction III) and 23,000 (particulate fraction) were found. The various antigenic preparations were used to immunize golden hamsters. Antibody was demonstrated in agar gel diffusion tests and in ELISA. Comparatively higher antibody response was found in animals immunized with whole antigen preparation, while fairly adequate responses were obtained with particulate fraction and subcellular fraction III.

INTRODUCTION

KNOWLEDGE about the nature of antigenic components of parasites, and the antigenic stimulation provided by them, is crucial for understanding host–parasite interaction. In the case of *Leishmania donovani*, even a partial characterization of antigenic components would be useful for effective utilization of parasite antigens in serodiagnosis and possibly for future use in vaccination experiments. The parasite is believed to have a large repertoire of non-specific and specific antigens[1,2]. The application of newer separation techniques has now made it possible to identify those antigens better, biochemically and immunologically. The various procedures currently in use for separating the antigenic components of *L. donovani* promastigotes include centrifugation[3] and sonic disruption[4].

In the present study an attempt was made to isolate the antigenic components of promastigotes. The isolated samples of whole antigen and various subcellular fractions were also partially characterized. The DNA, protein and carbohydrate contents of the soluble, particulate and subcellular fractions were estimated. Molecular weights of proteins in the various preparations were determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The antigenicity of the various antigen fractions was checked in agar gel diffusion tests, using serum from a kala-azar patient. The immunogenicity of such leishmanial antigen isolates was further established by immunizing golden hamsters and demonstrating antibodies against the various antigenic preparations in the immune sera by enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Promastigote-stage cells of *L. donovani* (NICD: CII strain) were harvested from the overlay of $BHMAEH_9$ medium and subjected to six successive washings in sterile normal saline containing gentamycin. The final deposit was resuspended in sterile, double-distilled water to 1/13–1/14 of the original volume[5]. The suspension was left overnight in the refrigerator and then examined microscopically to confirm complete parasite osmolysis. The osmolysed parasites were used as 'whole antigen'. One part of whole antigen was centrifuged at 105,000 *g* for 1 h to get soluble and particulate fractions. The remaining portion was subjected to successive centrifugations at 4000 *g* for 10 min to get subcellular fraction I (SCF I). Other subcellular fractions, SCF II and SCF III, were obtained following further centrifugation at 14,000 *g* for 20 min and at 105,000 *g* for 1 h respectively[4]. Each of these fractions was washed in sterile normal saline and resuspended in the same

diluent. The above antigen suspensions were further disrupted by ultrasonication at 30 MHz in five 1-minute bursts. Protein content of whole antigen and subcellular fractions was estimated according to the method of Lowry *et al*[6] using bovine serum albumin as the standard. Carbohydrate (hexoses) was estimated according to the method of Dubois *et al*[7] employing glucose as the standard. DNA content was determined by the Burton[8] method. SDS-PAGE was used to determine molecular weights of proteins in whole antigen and antigen fractions according to the method of Laemmli[9]. The marker proteins used were carbonic anhydrase (29,000), egg albumin (45,000), bovine albumin (66,000), phosphorylase (97,400), β-galactosidase (116,000) and myosin (205,000).

Healthy male golden hamsters weighing between 80 and 100 g were inoculated with whole antigen, or soluble, particulate or subcellular fraction. Each hamster was given four intraperitoneal injections, each containing 0.2 mg antigen protein, with intervals of 4 days. The agar diffusion technique was used to assess the antigenicity of leishmania antigens. ELISA was performed according to the method of Lin *et al*[10] with some modification. The anti-hamster IgG in this test was prepared according to the method of Sober *et al*[11].

Eight animals randomly selected from each group were bled on days 30, 45 and 60 after the last immunizing injection. The sera obtained from these blood samples were tested for anti-leishmania antibodies by ELISA.

RESULTS

Samples of whole antigen, and soluble, particulate and subcellular fractions showed well-defined precipitin lines against positive kala-azar serum (figure 1). The protein, carbohydrate and DNA contents of whole antigen and fractions are shown in table 1.

Table 2 shows results from the SDS-PAGE analysis of the various antigen fractions. The whole antigen, particulate fraction and SCF I were found

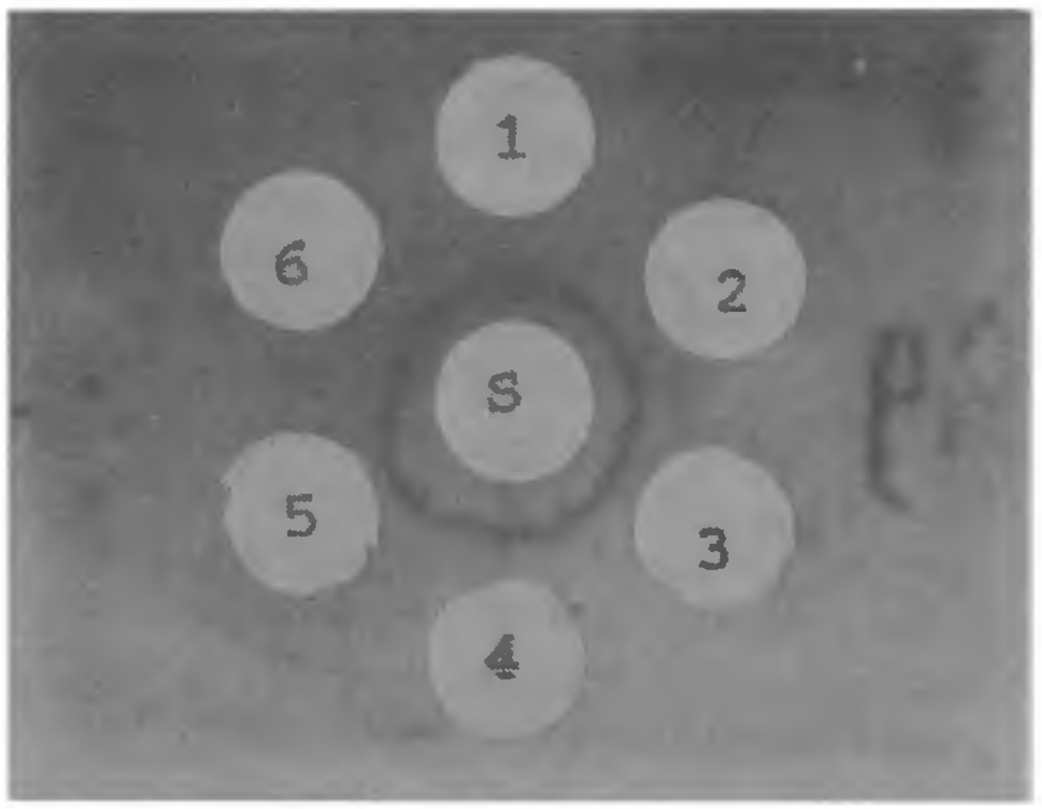

Figure 1. Precipitin reaction profile of antigen fractions versus kala-azar serum. Central well: Serum; Peripheral wells: (1) Whole antigen; (2) Soluble antigen; (3) Particulate antigen; (4) Subcellular fraction I; (5) Subcellular fraction II; (6) Subcellular fraction III.

Table 1 *Characterization of L. donovani promastigote antigens*

Antigen preparation	Protein (mg/ml)	Carbohydrate (mg/ml)	Protein to carbohydrate ratio	DNA (μg/ml)
Whole antigen	3.40	1.61	2.11	0.98
Soluble antigen	1.10	0.20	5.50	0.05
Particulate antigen	1.70	0.60	2.83	0.87
SCF I antigen	1.20	0.31	3.87	0.59
SCF II antigen	0.13	0.07	1.85	0.12
SCF III antigen	0.07	0.02	3.50	0.06

Table 2 *Molecular weights of L. donovani promastigote antigen proteins by SDS-PAGE*

Whole antigen	Soluble fraction	Particulate fraction	SCF I	SCF II	SCF III
102,920	50,880	87,850	65,880	85,310	117,160
69,780	21,160	56,210	43,410	35,350	53,070
41,560		23,580	32,100		21,880
26,290		17,100	21,270		

to resolve into four protein bands. SCF III resolved into three protein bands, while the soluble portion and SCF II resolved into only two bands.

The results of ELISA are shown in table 3. The maximum O.D. values were obtained with sera from animals immunized with whole parasite antigen at 45 days after the injection of the last immunizing dose. No antibodies were detected in the sera of control animals which received saline only.

DISCUSSION

Studies on *L. donovani* antigens have been mainly carried out on cell-free extracts of promastigotes[4, 12, 13]. In the present study attempts were made to obtain uncontaminated, cell-free extracts. Such extracts were prepared from 6-day-old cultures. The washed parasites were disrupted by osmolysis and subjected to differential centrifugation to isolate various fractions.

The biochemical characterization data clearly suggest that the various antigenic fractions of *L. donovani* promastigotes contain both protein and carbohydrate moieties. Other workers have also suggested a protein–polysaccharidic nature for *L. donovani* promastigote antigens[14]. Only proteinaceous antigens were able to elicit humoral antibodies, while the polysaccharidic antigens did not[15]. The methods employed for chemical characterization of antigenic isolates in this study were similar to those used by other workers for determination of protein and carbohydrate contents of immunologically active factor of *L. tropica*[16] and of antigenically active glycoproteins released by *L. donovani*[17]. Most of the earlier studies on the antigenicity of leishmania parasites were mainly confined to surface antigens of *L. donovani*. For example Lepay *et al*[18] showed the presence of three major antigenic proteins of molecular weight 65,000, 25,000 and 23,000 on the surface of *L. donovani*. The antigen with a molecular weight of 65,000 appeared to be glycoprotein in nature. It was shown that a deeply stained band in the SDS-PAGE pattern of *L. donovani* proteins was that of a pellicular membrane protein with a molecular weight of about 53,000. Probably this fraction was later recognized as a glycoprotein[15]. Ramosamy *et al*[19] also showed the presence of a similar band (53,000) on the outer surface of the membrane of *L. donovani* promastigotes. Four labelled proteins with apparent molecular weights of 65,000, 60,000, 50,000 and 26,000 were detected in both *L. donovani* and *L. chagasi* extracts[20]. Our results also show the presence of proteins with molecular weight in the region of 26,000 (whole antigen), 50,000 (soluble fraction), 65,000 (SCF I), 53,000 (SCF III) and 23,000 (particulate fraction). Further analysis of various antigen fractions tends to indicate that the bands were mostly glycoproteinaceous in nature. Since these antigen fractions are largely made up of glycoproteins, unglycosylated polypeptides perhaps represent only a small fraction of the total number of promastigote proteins.

In previous studies, the immunologic behaviour of leishmanial antigens was mostly demonstrated by simple slide precipitation tests or in agar gel diffusion plates[16]. In this study, the antigenicity of these fractions was shown by demonstrating antibodies in sera from experimentally immunized golden hamsters using ELISA. The highest O.D values in the ELISA were obtained with sera from animals immunized with whole antigen; the various fractions were somewhat less antigenic. But on a qualitative basis all the preparations showed well-defined precipitin bands on agar gel plates (figure 1).

Table 3 *O.D. values given by sera from golden hamsters immunized with various L. donovani antigenic fractions*

Animal groups	Days		
	30	45	60
Whole antigen	0.903 (0.602–1.204)	2.408 (2.107–2.709)	1.806 (1.505–2.107)
Soluble fraction	0	0.602 (0.301–0 903)	0.301 (0 –0.602)
Particulate fraction	0.903 (0.602–1.505)	2.107 (1.505–2.709)	1.806 (1.505–2.408)
SCF I	0.301 (0 –0.602)	1.204 (0.903–1.505)	0.903 (0.602–1.204)
SCF II	0	0.903 (0.602–1.204)	0.602 (0 –0.903)
SCF III	0.301 (0 –0 602)	1.505 (1.204–2.107)	1.204 (0.602–1.806)
Non-immune serum (saline control)	0	0	0

O.D. was read at 400 nm; each value is mean of O.D. values given by 8 different sera; values in parentheses give range of O.D. values.

The apparent differences in the immunologic response to the various preparations reflect the degree of antigenic stimulation the latter were able to provide. Such inherent variations/differences in their antigenicity are in fact well represented in the results of this investigation. Rassam and Al-Mudhaffar[21] employed micro-ELISA to estimate *L. donovani* soluble antigen using antisera raised in rabbits. Among the different techniques which have been used in diagnosis of kala azar, micro-ELISA was found to be more sensitive than immunoelectrophoresis or couhterimmunoelectrophoresis[22]. The use of ELISA for diagnostic purposes has also shown promising results[3, 23].

These and similar studies will be helpful in future attempts towards the development of an effective vaccine against leishmaniasis.

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