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Antigenic Relationship among Kinetoplastid Flagellates: *Herpetomonas, Leishmania* and *Bodo*

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ABSTRACT

The antigenic relationship(s) amongst kinetoplastid flagellates viz. *Leishmania donovani* promastigotes, *Bodo indica* n. sp. and *Herpetomonas indica* n. sp. have been evaluated using immunoelectrophoresis, indirect fluorescent antibody test, enzyme-linked immunosorbent assay, sodium dodecyl sulphate-polyacrylamide gel electrophoresis and electroimmunotransfer blot assay. Antisera raised against the three organisms were tested with homologous and heterologous antigens. In all the assays, common antigens against three flagellates have been demonstrated against the homologous and heterologous antibodies. However, a more closer antigenic relationship between *H.indica* n. sp. and *L. donovani* antigens and antibodies was observed in all tests than *B. indica* n. sp. antigen and antibodies to *H. indica* n. sp. or *L. donovani* antigen and antibodies. These findings will be very useful in studies on phylogenetic relationship between Bodonina and Trypanosomatina of order Kinetoplastida.

INTRODUCTION

The order Kinetoplastida consists of two suborders namely Trypanosomatina (monoflagellum) and Bodonina (biflagella). The genera *Herpetomonas* and *Leishmania* belong to suborder Trypanosomatina whereas *Bodo* belongs to Bodonina. The first study by Noguchi (1926) on serological relationships amongst the kinetoplastid flagellates have served as a basis for consideration of species and genus affinities. *Crithidia fasciculata* has been shown cross-reacted with *C. oncopelti, C. luciliae* or other *Crithidia* sp. from Euryophthalmes (McGhee 1963). On the basis of agglutination test three strains of *C. fasciculata* and one strain of *C. oncopelti* were found immunologically similar (Vitetta and Guttman 1967). The anti *Leptomonas pessoai* serum showed cross-reactivity with *T. cruzi* as detected by immunodiffusion and rosette technique (Souza et al. 1974). Sera

from patients with Chagas' disease agglutinated culture forms of *C. oncopelti* and *T. cruzi* epimastigotes (Vattuone and Yanovsky 1971). Antigenic cross-reactivity has been reported between *L. donovani* and *Crithidia* spp. (Hedge et al. 1979, Lopez-Brea 1979). Immunological cross-reactivity between *Trypanosoma cruzi* and insect trypanosomatids have been demonstrated by different immunological assay (Lopes et al. 1981). The membrane specific monoclonal antibodies generated against promastigotes of New-World *Leishmania* species showed cross-reactivity with *T. cruzi* antigen by immunoblot and enzyme-linked immunosorbent assay (Williams et al. 1986). Six monoclonal antibodies specific to flagellar proteins of *Leishmania* cross-reacted with members of family Trypanosomatidae *Endotrypanum*, *Trypanosoma* (Ismach et al. 1989). The proteases from *L. major* promastigotes and *H. samuelpessoai* were shown to share several common features such as, glycosyl-phosphatidylinositol (GPI)-anchor, surface location and activity, sensitivity to 1, 10-phenanthroline and peptide bond preference (Schneider and Glaser 1993).

The nucleotide pdJ was detected in *T. brucei* and other kinetoplastid species (Gommers-Ampt et al. 1991). Partially RNA edited molecules for the cytochrome-c oxidase subunit III (COIII) transcript shows similarity in *T. brucei* (Feagin et al. 1988) and *H. samuelpessoai*, *H. mariadeanei*, *H. m. muscarum*, *L. tarentolae* and *C. fasciculata* (Landweber and Gilbert 1993); *H. megaseliae* and *H. mariadeanei* (Landweber et al. 1993). Recently, similar pan-editing genes (MURF4, ND7 and COIII) were isolated from *T. brucei*, *T. cruzi*, *L. tarentolae*, *C. fasciculata*, *H. muscarum*, *Blastocrithidia culicis* and *Trypanoplasma borreli* (Maslov et al. 1994). All these facts suggest the existence of common antigens or genes among kinetoplastid flagellates. However, none of the earlier workers have studied the immunology of kinetoplastid flagellates in a comparative way and especially, with Bodonina (biflagellate) to Trypanosomatina (monoflagellate). In the present communication, efforts have been made to evaluate the antigenic similarities among the kinetoplastid flagellates using serological tests such as immunoelectrophoresis (IEP), indirect fluorescence antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroimmunotransfer blot (EITB) assay.

MATERIALS AND METHODS

Organisms

Herpetomonas indica n. sp. (De 1992), isolated from the hindgut of *Chrysomya megacephala* (bottle green fly), was maintained at 25°C in modified Tobie's medium (Ghosh et al.1987). However, for antigen preparation, organisms were cultured in a tissue culture medium containing hemin, biotin, L-glutamin and folic acid (Kar et al.1990).

Leishmania donovani, isolated from a kala-azar patient by bone marrow aspiration, was maintained in modified Tobie's medium at 25°C. Antigen was prepared from *L. donovani* promastigotes in defined medium (Kar et al. 1990).

Bodo indica n. sp. (De 1992), isolated from the hindgut of *Lethocerus indicus* (giant water bug), was routinely maintained at 25°C in modified Tobie's medium without blood.

Preparation of antigen

Each flagellate was harvested by centrifugation at 5,000 xg for 30 min at 4°C, washed three times in phosphate-buffered-saline (PBS, pH 7.2) and subjected to sonication (Labsonic 2000 ultrasonicator, B. Braun Melsungen AG) for about 3 min (six bursts, each of a 30 sec duration) in an ice bath. Homogenates were centrifuged at 10,000 xg for 30 min at 4°C and the clear supernatants were used as soluble antigen. Protein contents were estimated according to the method of Bradford (1976). Phenyl methyl sulphonylfluoride and leupeptine at a final concentration of 2 mM and 2 µg/ml respectively were added to antigen as protease inhibitor.

Immunization of rabbits

Albino rabbits, weighing 1.5 kg to 2 kg were immunized with *H. indica* n. sp., *L. donovani* and *B. indica* n. sp. soluble antigen(s). A total of six immunization doses were administered to each rabbit of which the first three immunizations were given subcutaneously at four different sites with equal volume of Freund's complete adjuvant (Gibco) at 10 days interval, and the last three doses were injected intravenously with antigen alone. Sera from these animals were collected after 10 days of last immunization. Prior to immunization sera were collected from all the rabbits for their subsequent use as healthy controls. All the sera were stored at -20°C and heat inactivated at 56°C for 30 min prior to use.

Adsorption experiments

Formaldehyde (2%) fixed and thoroughly washed (in PBS, pH 7.2) flagellates ($1x10^7$cells) were suspended in 1 ml serum and left overnight at room temperature with constant agitation. The unadsorbed serum was collected after centrifugation at 5,000 xg for 10 min. The clear serum was used in the studies or cross-reactivity amongst the homologous and heterologous antigen-antibody assay.

Immunoelectrophoresis

Immunoelectrophoresis test was done according to Pan (1986). In brief, 7 ml of 1% agarose (Sigma) in Tris-barbital buffer (pH 8.6) was poured onto a glass slide (8.4x7.4 cm). Antigen wells and antibody troughs were punched as shown in Figures 1a-1c. Each antigen well received 16 µl of soluble antigen containing 120 µg of antigenic proteins and electrophoresis was carried at 4°C in Tris-barbital buffer (pH 8.6) under constant current of 1.5 mAmp/cm (horizontal LKB 2117 multiphor II electrophoresis, Sweden) for 4 hr. A 175 µl of serum was added to the antibody trough and the slides were incubated at room temperature (25-30°C) for 24 hr to 48 hr in a humid chamber.

Indirect fluorescence antibody test

This test was performed according to the method described by Shaw and Voller (1964). Briefly, active cultures (72 hr old) showing exponential growth were washed three times with PBS (pH 7.2) and fixed in cold methanol for 15 min. Fixed cells were washed five times with PBS (pH 7.2) and finally a cell suspension of 100 organisms per high power field (40 x) was adjusted. Six drops of antigen suspensions were added on

clean microscope slides. Once the antigen was air dried, 20 to 30 µl of two fold serial dilutions of test serum (immunized or pre-immune) was delivered to each drop of antigen and incubated at 37°C for 45 min in a humid chamber. Excess antibody was washed and 20 to 30 µl of working dilution of anti-rabbit IgG fluorescein isothiocyanate (FITC) (Sigma) was added to each drop of antigen antibody spots and the slides were incubated as before. The concentration of anti-rabbit FITC for *H. indica* n. sp. and *L. donovani* was 1:40 and 1:160 for *B. indica* n. sp. Finally, the slides were rinsed in PBS (pH 7.2), air-dried and mounted in 90% glycerol buffer (pH 7.2). Fluorescence was examined with a fluorescence photo microscope (Carl Zeiss, Germany).

Enzyme linked immunosorbent assay

The technique for performing the test was essentially the same as described by Hommel et al. (1978). Briefly, 100 µl of 20 µg/ml working dilution of soluble antigens of each parasites prepared in carbonate-bicarbonate buffer (0.05 M, pH 9.6) was added to each well of 96 flat bottom microtiter ELISA plate. The plates were incubated overnight at 4°C and next morning washed three times with 0.85% saline containing 0.05% tween 20 (Sigma). A 100 µl of appropriately diluted (1:200) serum was added in duplicate wells and the contents were incubated for 90 min at 37°C. Excess serum was washed as above, and 100 µl of optimally diluted (1:1000 in PBS, pH 7.2) conjugate (horse raddish peroxidase anti-rabbit IgG, Fab part, Sigma) was added to each well and incubated as above. After washing, 100 µl of freshly prepared substrate solution (orthophenyl diamine in 0.2 M phosphate-citrate buffer, pH 3.7, containing 0.02% H_2O_2, v/v) was added to each well and the reaction was allowed to proceed for exactly 15 min in dark. Reaction was stopped by addition of 25 µl of 5 N H_2So_4 and colour intensity of each well was recorded at 492 nm wave length in an ELISA reader (Dynatech Laboratories, Inc.) using substrate control as blank.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Using the standard technique of Hames and Rickwood (1986) 3% of stacking gel and 5-20% continuous gradient gel was used in separation of hundred microgram of antigenic proteins of three parasites. Electrophoresis was carried out at 25 mAmp constant current and terminated when the dye front migrated to 1-2 cm of the gel. Gel was stained with 0.2% (w/v) Coomassie brilliant blue (KOCH-light).

Electro immunotransfer blot

The method used for EITB was essentially the same as described by Das et al. (1993). In brief, all the three antigens were first separated in 5-20% SDS-PAGE. Separated proteins were transferred to nitrocellulose membrane (0.45 µm pore size) using transblot buffer in a transblot cell (Bio-Rad Laboratories, Richmond, CA) at 4°C for 5 hr. Once the proteins were transferred to nitrocellulose membrane they were incubated in blocking solution (3% bovine serum albumin, prepared in TBS, 10 mM Tris and 0.15 M saline) for overnight at 4°C with constant shaking. Next morning the membrane was washed two times with TBS and incubated with 1:1000 dilution (found optimal in standardization process) of *H. indica* n. sp. antibody at 37°C for 90 min with continuous agitation. The membrane was washed as above and exposed to peroxidase-conjugated anti-rabbit IgG

(1:1000 dilution in TBS-BSA solution) for 60 min at 37°C. Colour reaction was developed in substrate solution (5 mg/ml of 3-3' diaminobenzidine tetrahydrochloride, DAB, in TBS, 0.02% H_2O_2) and reaction was stopped by rinsing the membranes in deionized water and the blot dried.

RESULTS

Immunoelectrophoresis

The hyperimmune rabbit serum raised against *H. indica* n. sp. (HIRS-H) showed 13 precipitin bands (Nos. 1-13) with the homologous antigen and these bands appeared to

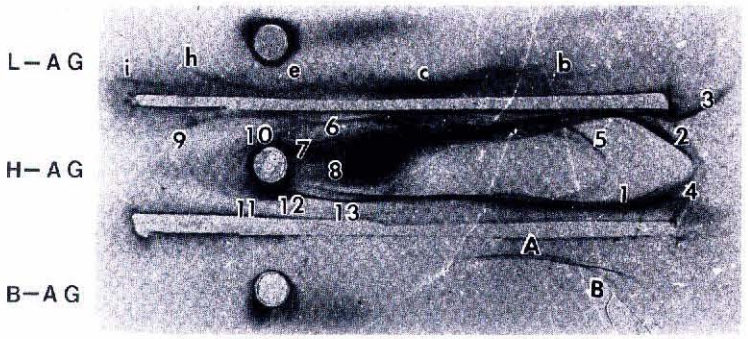


Fig. 1a. Single dimension immunoelectrophoretic reaction of anti *H. indica* n. sp. antibody (HIRS-H) (175 µl undiluted) against *B. indica* n. sp. (B-AG), *H. indica* n. sp. (H-AG) and *L. donovani* (L-AG) antigen in 1% agarose. The antigens (16 µl containing 120 µg of protein) were electrophoresed at a constant current (1.5 mAmp/cm) for 45 min.

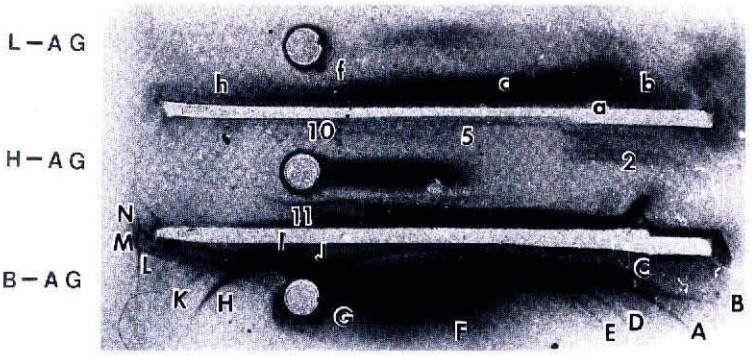


Fig. 1b. Single dimension immunoelectrophoretic reaction of anti *B. indica* n. sp. antibody (HIRS-B) (175 µl undiluted) against *B. indica* n. sp. (B-AG), *H. indica* n. sp. (H-AG) and *L. donovani* (L- AG) antigen in 1% agarose. The antigens (16 µl containing 120 µg of protein) were electrophoresed at a constant current (1.5 mAmp/cm) for 45 min.

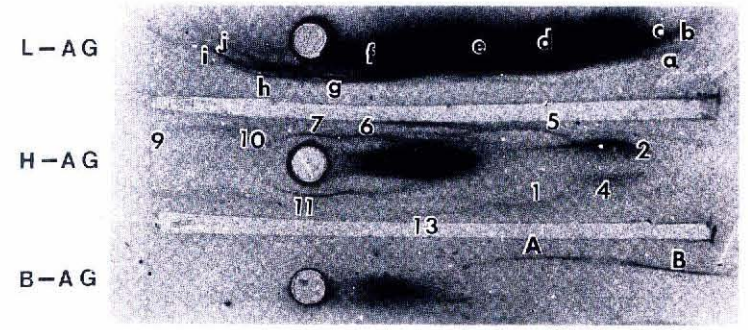


Fig. 1c. Single dimension immunoelectrophoretic reaction of anti *L. donovani* antibody (HIRS-L)(175 µl undiluted) against *B. indica* n. sp. (B-AG), *H. indica* n. sp. (H-AG) and *L. donovani* (L-AG) antigen in 1% agarose. The antigens (16 µl containing 120 µg of protein) were electrophoresed at a constant current (1.5 mAmp/cm) for 45 min.

extend from the anodal end to the cathodal region. The strongest, probably compound band (Nos. 1, 2 and 3) migrated towards the cathode. However, the other prominent bands 6, 7, 8, 9, 10, 11 and 12 were observed close to the antigen well (Fig. 1a). The

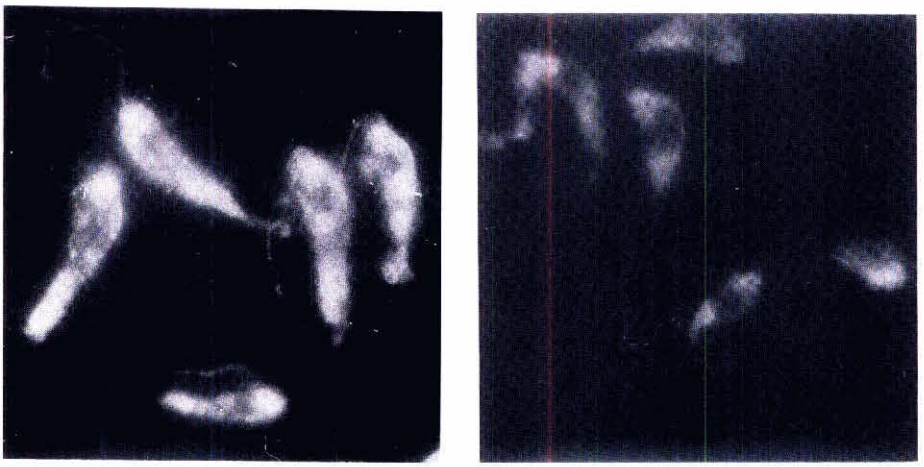
reaction between HIRS-H and *B. indica* n. sp. antigen showed only two precipitin arcs which were labeled as A and B, they were found towards the cathodal ends. *Leishmania donovani* antigen against immunized rabbit serum to *H. indica* n. sp. showed 5 weak bands viz. b, c, e, h and i.

The hyper immune serum to *B. indica* n. sp. (HIRS-B) showed 14 precipitin arcs (Nos. A-N) when reacted with homologous antigen (Fig. 1b). The precipitin bands appeared throughout the gel from anode region to cathodal end. The five bands viz. A, B, C, D and E were prominent than others and thereby implying their homology with those noted in HIRS-L and HIRS-H against *B. indica* n. sp. antigen. The precipitin reactions of immune serum to *B. indica* n. sp. against *H. indica* n. sp. and *L. donovani* antigen were generally weaker (bands 2, 5, 10, a, b, c, f and h).

The hyper-immune rabbit serum raised against *L. donovani* (HIRS-L) showed 10 precipitin bands (Nos. a-j) with homologous antigen (Fig. 1c). The prominent precipitin bands were seen both at the cathodal end (Nos. c, d and e) and anodal end (Nos. g, h, i and j). Lesser number of bands were observed when HIRS-L was tested against heterologous antigen i. e., *H. indica* n. sp. and *B. indica* n. sp. antigens. The most interesting observation was the absence of precipitin bands c, h and j in the reaction between HIRS-L and *H. indica* n. sp. antigen. Similarly, the reaction between HIRS-L and *B. indica* n. sp. antigen showed absence of bands in cathodal region (Fig. 1c).

Indirect fluorescence antibody test

In IFA test bright fluorescence was observed throughout the body and flagellum when



a b

Fig. 2. Photomicrographs showing the effect of antigen-antibody reactions in IFAT using *B. indica* n. sp. as antigen. The active cultures (72 hr old) after fixation were reacted with FITC following the incubation with antibody. **a**. Homologous antigen-antibody reaction using *B. indica* n. sp. antigen and *B. indica* n. sp. antibody. **b**. Heterologous antigen-antibody reaction using *B. indica* n. sp. as antigen and *L. donovani* antibody. Magnification 1000x.

L. donovani and *H. indica* n. sp. were treated with either homologous or heterologous serum. In contrary, only homologous antibody showed bright fluorescence on body and one flagellum of *B. indica* n. sp. (Fig. 2a). However, the heterologous antibodies i. e., *L. donovani* and *H. indica* n. sp. antisera produced weak surface fluorescence and no flagellar fluorescence against *B. indica* n. sp. antigen (Fig. 2b).

Enzyme linked immunosorbent assay

To detect the cut off point for IgG antibodies in ELISA test the positive and negative sera were assayed at different dilutions viz., 1:200, 1:400, 1:800 and 1:1600. A 1:200 dilution was found optimal in the sense that it completely separated out the negative serum from the positive ones. The result of ELISA using 1:200 dilution is presented in Fig. 3. At this dilution, all the positive (homologous) sera showed mean O. D. >0.40 values whereas, the preimmune (control) sera showed mean O. D. <0.10 values. In heterologous system, *L. donovani* antigen revealed greater O. D. values (mean O. D. 0.28) with *H. indica* n. sp. antibody than with the *B. indica* n. sp. antibody (mean O. D. 0.22). Similarly, when *H. indica* n. sp. antigen was reacted with *L. donovani* and *B. indica* n. sp. antibodies, former showed greater O. D. value (mean O. D. 0.34) than the latter (mean O. D. 0.18). Reaction of *B. indica* n. sp. antigen was observed more with *L. donovani* antibodies (mean O. D. 0.30) than *H. indica* n. sp. antibodies (mean O. D. 0.16).

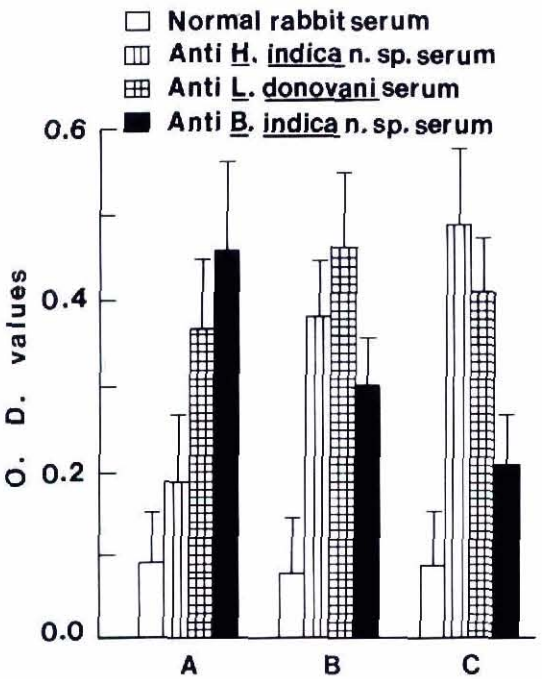


Fig. 3. Effect of antigen-antibody reaction in ELISA test. The active cultures (72 hr old) were sonicated and centrifuged. The supernatant was used as antigen (20 µg/ml). Following the incubation with antiserum (1:200 dilution) and HRP-conjugate (1:1000 dilution) the colour was developed using OPD as substrate. The OD values were taken at 492 nm. The results shown are the mean ± SE from three independent experiments. A. *B. indica* n. sp. antigen. B. *L. donovani* antigen. C. *H. indica* n. sp. antigen.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Electro immunoblot analysis

The results of SDS-PAGE and immunoblot analysis are shown in Fig. 4. In SDS-PAGE the common polypeptides among the three flagellates were observed in the molecular weight region at 68 kDa, 60 kDa, 56kDa, 43 kDa, 42 kDa, 38 kDa, 33 kDa, 30 kDa, 25 kDa, 23 kDa, 18 kDa, 16 kDa, 15 kDa and 14 kDa (Fig.4, arrow head). However, the common immunoreactive polypeptide against *H. indica* n. sp. antibody was observed at 60 kDa in all the antigens (Fig. 4, arrow). Homologous antigen-antibody (*H.*

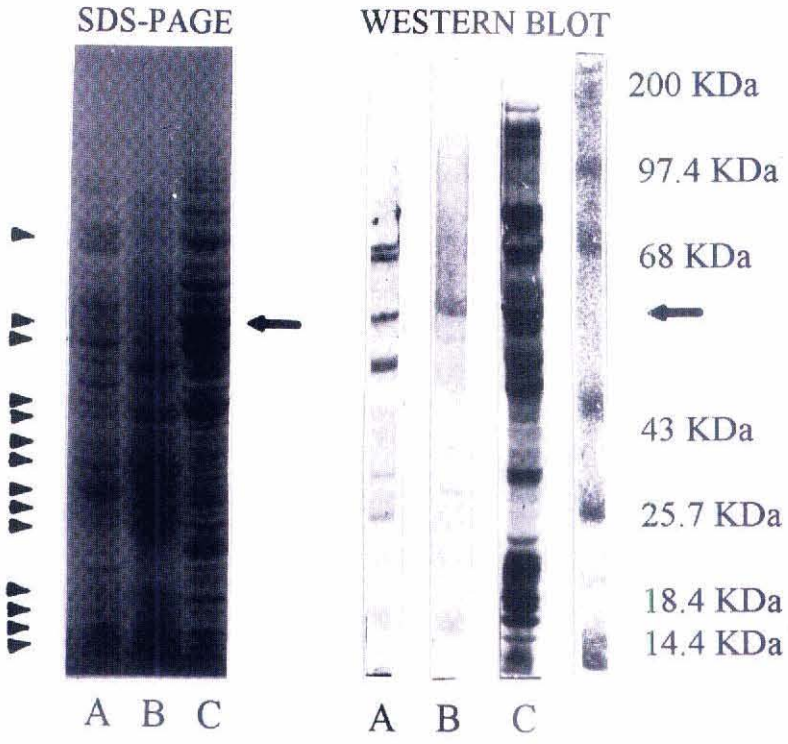


Fig. 4. SDS-polyacrylamide gel electrophoresis and immunoblot analysis of 3 kinetoplastid flagellates. One hundred µg of soluble antigenic proteins of A, *L. donovani*; B, *B. indica* n. sp.; and C, *H. indica* n. sp. were analyzed by SDS- PAGE on 5-20% (w/v) gradient gels. Gels were either stained with Coomassie brilliant blue to visualize protein bands or transferred electrophoretically to nitrocellulose membrane for immunoblot analysis. The membrane was stained with an antibody to *H. indica* n. sp. (1:100 dilution) using DAB as chromogen and H_2O_2 as substrate. The size of the molecular weight markers is indicated on the right side of the gel. The common band both in SDS-PAGE and immunoblt among three flagellates is indicated by arrow. The common bands in SDS-PAGE among three flagellates are indicated by arrow head.

indica n. sp. antigen- antibody) showed immunoreactive polypeptides in the range of 190 kDa to 14 kDa regions with major polypeptides at 170 kDa, 150 kDa, 84 kDa, 68 kDa, 60 kDa, 52 kDa, 30 kDa and 20 kDa to 14 kDa molecular masses (Fig. 4, Western blot, lane

C). The common immunoreactive polypeptides between *H. indica* n. sp. antibody and *L. donovani* were observed at 67 kDa, 60 kDa, 50 kDa and 33 kDa (Fig. 4, Western blot, lane A). On the other hand, *H. indica* n. sp. antibody and *B. indica* n. sp. antigen showed common polypeptides at 54 kDa, 40 kDa and 30 kDa (Fig. 4, Western blot, lane B).

DISCUSSION

The present study clearly demonstrated the antigenic similarities in the flagellates of three genera namely, *Leishmania*, *Herpetomonas*, and *Bodo*. This is perhaps the first report where immunological relationship between the members of the suborder Bodonina was evaluated with the well-established members of the suborder Trypanosomatina on the basis of IEP, IFAT, ELISA, SDS-PAGE and EITB. Data obtained from all the experiments suggest that antigenic molecules of *L. donovani* and *H. indica* n. sp. are more closely related than antigenic molecules of *B. indica* n. sp.

Immunoelectrophoresis has been used for serodiagnosis of kala-azar (Rezai et al. 1977, Mancianti and Meciani 1988, and Mittal et al. 1991) and for the detection of *T. evansi* antibody (Raina et al. 1986). In IEP studies major differences amongst the three antigens were analyzed on the basis of presence or absence of precipitin bands in heterologous system compared to homologous system. The results of this analysis definitely suggest the presence of common antigens among the three flagellates. The precipitin band appears to be shared by all the antigens, however, judging from the intensities and presence or absence of bands, there seem to be quantitative and perhaps qualitative differences (Pan 1986) among these three organisms.

Both ELISA and IFAT have been used for serodiagnosis of leishmaniasis and trypanosomiasis by different workers (Walton et al. 1972, Rezai et al. 1986, Anthony et al. 1979, Mancianti and Meciani 1988 and Mittal et al. 1991). In our present study with ELISA test, *H. indica* n. sp. antigen and *L. donovani* antigen always showed greater O. D. values with either *L. donovani* or *H. indica* n. sp. antibody rather than *H. indica* n. sp. antigen to *B. indica* n. sp. antibody or *B. indica* n. sp. antigen to *H. indica* n. sp. antibody (Fig. 3). These results once again support the view that *H. indica* n. sp. and *L. donovani* have more closely related antigen(s) than *B. indica* n. sp. antigen(s). These observations probably suggest that the sequences between *L. donovani* and *H. indica* n. sp. for generating antibody response are more conserved than *B. indica* n. sp. antigen. However, this needs further investigation. The surface analysis in immunofluorescence assay showed a cross-reactivity within the parasites. The pre adsorption of antigen against three parasites drastically reduced the fluorescence with heterologous and homologous systems. These observations strengthen the view that these flagellates have common antigen(s). The results of HIRS to *B. indica* n. sp. showed fluorescence on whole body surface and only one flagellum of *B. indica* n. sp. This can be explained by the fact that the flagellar protein (non-fluoresced) was precipitated with particulate antigen. In this respect it is noted that we used only soluble antigen (supernatant of 10,000 xg) as this antigen was found more immunoreactive than the particulate antigen.

In *H. samuelpessoai*, *T. cruzi* and *L. donovani* a 43 kDa polypeptide has been reported which was co-migrated with skeletal muscle actin has (Pan 1986, Mortara 1989). A similar polypeptide was noticed in all the antigens in SDS-PAGE analysis, thereby, suggesting the existence of actin like protein in these three organisms. However, these observations need further investigation. The presence of a 68 kDa polypeptide can be correlated with the 68 kDa protein which was discussed by Chang and Fong (1983) in *L. mexicana*.

The immunoprecipitation after radiolabelling of *Leishmania* promastigotes showed 23 kDa, 42 kDa and 68 kDa polypeptides (Chang and Fong 1983, Pan 1986). The present EITB studies showed a 68 kDa immunoreactive polypeptide both in *L. donovani* and *H. indica* n. sp. However, the uncommon polypeptides noticed with each organism may perhaps suggest the variation in or specificity of each group. A common immunoreactive polypeptide at 60 kDa in these organisms suggests a common antigenic epitope. Presence of more common immunoreactive polypeptides with *H. indica* n. sp. antigen to *L. donovani* antibody or *L. donovani* antigen to *H. indica* n. sp. (data not shown) once more suggests the closer relationship in the antigenic make up of these two parasites. On the other hand, *B. indica* n. sp. antigen showed more common immunoreactive polypeptides with *L. donovani* antibody rather than *H. indica* n. sp. antibody (data not shown), which implies the presence of more common antigenic epitopes between *B. indica* n. sp. and *L. donovani* antigen than *B. indica* n. sp. and *H. indica* n. sp. Interestingly, it was also noticed that the immunoreactive polypeptides in *H. indica* n. sp. antigen ranges from 190 kDa to 10 kDa, in *L. donovani* 95.5 kDa to 20 kDa and in *B. indica* n. sp. 75 kDa to 20 kDa when reacted with homologous antibodies. These findings tend to speculate the idea that trypanosomatid (monoflagellate) is more evolved than bodonine (biflagellate).

The results of this study would have been more meaningful if the monoclonal antibody could have been raised at least against one parasite and would have been used for the cross-reactivity analysis among three genera. However, the results with polyclonal antibody certainly suggest the antigenic relationship among the three parasites of order Kinetoplastida. These findings will be very useful for future research workers in drawing a phylogenetic relationship tree between the three parasites for future evolution studies.

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