

## IN VITRO EXOANTIGEN FROM HERPETOMONAS SAMUELPESSOAI WHICH PROTECTS MICE AGAINST TRYPANOSOMA CRUZI INFECTION

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### SUMMARY

This paper describes the extraction, purification and characterization of *Herpetomonas samuelpessoai* exoantigen. The antigen was obtained from a complex medium after 48 hours incubation at 28°C and characterized as a protein with a molecular weight of 58,800, having pH 4.2 as isoelectric point, without proteolytic activity and an  $E_{280nm}$  value of 39,900. Mice immunized by this exoantigen show a considerable protection against *T. cruzi* challenge.

INDEX KEY WORDS: *Herpetomonas samuelpessoai*; exoantigen; *Trypanosoma cruzi*; immunity to *T. cruzi*.

RUNNING HEAD: Exoantigen from *Herpetomonas samuelpessoai*

### INTRODUCTION

SOUZA & ROITMAN<sup>17</sup> reported a partial protection against *Trypanosoma cruzi* infection in mice inoculated with viable culture forms of *Herpetomonas samuelpessoai*, earlier named *Leptomonas pessoai*<sup>12</sup>. However the disrupted forms of this microorganism were not able to elicit resistance against *T. cruzi*<sup>16</sup>. This fact would suggest that a soluble exoantigen produced by the living cells such as those detected in the surface coat of *T. brucei* and in other trypanosomatids<sup>7,9,22</sup> may participate in the development of resistance in immunized animals.

Our present findings are related to the "in vitro" exoantigen production by *Herpetomonas samuelpessoai* and its isolation and characterization. This exoantigen was also assessed in protection experiments against *T. cruzi* challenge in mice.

### MATERIAL AND METHODS

**Protozoa:** *Herpetomonas samuelpessoai*, isolated from *Zelus leucogrammus* in the Instituto de Patologia Tropical (Universidade Federal de

Goiás)<sup>8</sup>; *Trypanosoma cruzi* (CL strain) provided by Drs. I. Roitman and N. Alvarenga (Universidade de Brasília) and *Trypanosoma hastatus hastatus* from the Instituto de Patologia Tropical (Universidade Federal de Goiás)<sup>1</sup>.

**Culture media** — LIT (liver infusion tryptose) was prepared as described by YAEGER<sup>3</sup> with the following composition (g/l): NaCl 4.0g; KCl 4.0g; Na<sub>2</sub>HPO<sub>4</sub> 8.0g; tryptose 5.0g; liver infusion 5.0g; glucose 2.0g; 10% bovine serum and this mixture was heated at 68°C for 60 minutes with shaking. Hemoglobin (2%), crystallized penicillin (50 U/ml) and streptomycin (100 µg/ml) were added and the pH was adjusted with 2.0N HCl to 7.2. This medium was sterilized by filtration in a "Seitz" filter with an EKS membrane with positive pressure.

**Complex medium** — It was prepared with the following composition (g/l): tryptose 5.0g; yeast extract 7.0g; glucose 15.0g; hemin 20.0mg; folic acid 2.0mg. This medium was adjusted to pH 7.0 with NaOH and autoclaved for 20 minutes at 120°C.

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**Maintainance of organisms** — *H. samuelpessoai* and *T. hastatus hastatus* were grown in LIT medium at 28°C for 2 and 15 days respectively. After growth, they were kept in the cold room. Every 20 days they were transferred to a new medium. *Trypanosoma cruzi* (CL strain) was maintained in a albino mice (18-20g) by weekly transfers.

**Immune sera preparation** — The *T. hastatus hastatus* antigen was prepared as follows: the microorganisms were grown in LIT medium at 28°C for 48 hours; the cells were then collected by centrifugation at 1500g and washed 3 times with 0.15M NaCl. The cell concentration was estimated by counting in a Neubauer's chamber and diluted to  $8 \times 10^8$  cells/ml in distilled water. This suspension was frozen and thawed 5 times during 5 days and the cell rupture completed by cycles of 30 seconds sonication followed by 30 seconds interval during 5 minutes in a Bronson Sonifier Cell Disrupter. The cell fragments and intact cells were separated by centrifugation. The protein concentration in the supernatant was adjusted to 3mg/ml and sterilized by filtration in Millipore membrane (0.45 $\mu$ ).

The antigenic emulsion was prepared by mixing 1 ml antigen plus 1 ml dead flagellate suspension ( $2 \times 10^6$  cells in formalin) and 2 ml Freund's adjuvant.

The immune serum was prepared as described by COOMBS & GELL<sup>6</sup>. The New Zealand rabbits (3 kg body weight) received, in the first application, an intramuscular injection of 1 ml antigenic emulsion in each gluteal region. Fifteen days later, an equal volume was injected in each scapular muscle. On the 21<sup>st</sup> day, 10% concentration of the initial antigenic emulsion was injected intraperitoneally; after which, 0.5 ml of 1% diluted antigenic solution was injected every day intravenously for 5 days. Seven days after the last injection, the rabbits were bled and their sera collected and pooled.

**Purification of exoantigen from the cultivated media** — After 48 hours incubation of *H. samuelpessoai* in the complex medium with shaking at room temperature, each culture was checked by microscopy for possible contamination with other microorganisms. The cells were separated by centrifugation and the supernatant was fractionated by protein precipitation with solid ammonium sulfate. Two fractions (0-50% and 50-90% saturation) were collected and, after exhaustive dialysis, they were lyophilized.

The material precipitated at 50-90% ammonium sulfate saturation, which gave a positive reaction in the immunodiffusion test, was fractionated in a Sephadex G-75 column (2.6 x 40 cm) equilibrated in 0.05M phosphate buffer containing 0.2M KCl and 0.02% sodium azide at pH 7.0. For each chromatography 300 mg of lyophilized material was applied; the flow was calibrated to 25 ml/hour and 3 ml fraction were collected after recording the absorbance at 280nm.

The pool of fractions, which gave a positive reaction in the immunodiffusion test, after dialysis and lyophilization, was gel filtered again in a Sephadex G-150 column equilibrated with the same buffer system and the flow calibrated to 15ml/hour. The appropriate fraction was dialyzed, lyophilized and stored at -18°C.

**Polyacrylamide gel electrophoresis** — The simplified method described by CLARKE<sup>5</sup> was used with 7.5% gel concentration. The polymerization in 0.6 x 8.0cm tubes was carried out with TEMED, riboflavin and U.V. light. The buffer in both electrode chambers was 5mM Tris-Glycine, pH 8.1 and the run was made at 2mA/tube at 4°C temperature. The protein concentration applied was 100  $\mu$ g per tube. After 20 minutes run, the gels were stained with Amidoblack and destained with 7.0% acetic acid. The possible glycoprotein character of the exoantigen was analysed by staining the gel with Alcian Blue according to WARDI & MICHOS<sup>19</sup>.

**Isoelectric focusing** — Two tubes (0.6 x 10 cm) containing 8% polyacrylamide gel and 2.0% Ampholine (3-10 pH range) were used. The upper chamber (cathodic) was filled with 0.05M phosphoric acid and the lower (anodic) with 0.05M triethanolamine. Samples of 400  $\mu$ g protein dissolved in 10% sucrose and 2% Ampholine solution were applied to the top of the gel and overlaid by 10% sucrose and 2% Ampholine solution. The focusing was carried out at constant voltage for 4 hours, by setting the current to 2 mA/tube in each hour. After running, one gel was fixed in trichloroacetic acid and stained with Coomassie Brilliant Blue as described by CHRAMBACH<sup>4</sup> and the other gel was sliced in 1cm segments to determine the pH gradient by elution in double distilled water for 2 hours and by measuring the pH in a digital potentiometer.

**Evaluation of molecular weight by hydrodynamic parameters** — The molecular weight de-

termination was made by gel filtration in Sephadex G-150 column (2.6 x 40cm) equilibrated with 0.05M phosphate buffer containing 0.2M KCl, 0.02% azide, pH 7.0 at room temperature. The flow was calibrated to 15ml/hour and the void volume was determined with Blue Dextran. The column calibration was made with the following standard proteins: bovine serum albumin, egg albumin, alpha-chymotrypsin and myoglobin.

**Protease activity** — To test possible proteolytic activity of exoantigen, the caseinolytic method of KUNITZ<sup>10</sup> was used. A series of 13 tubes containing different amounts of purified exoantigen (2 to 200 µg/tube) in 0.1M phosphate buffer pH 7.0 was incubated for 20 minutes with 1% heat denaturated casein. After this time, 3 ml of 5% trichloroacetic acid was added and filtered after standing 30 minutes at room temperature. The proteolytic activity was correlated with 280nm absorbance of filtered solution.

**Absorption spectrum** — The purified and lyophilized exoantigen was dried on P<sub>2</sub>O<sub>5</sub> in a desiccator for 12 days. The absorbance of a solution containing 0.972 mg/ml in 0.05M phosphate, 0.2M KCl, pH 7.0 buffer was read in a ZEISS PMQ II spectrophotometer.

**Agar immunodiffusion method** — It was performed as described by OUCHTERLONY<sup>11</sup>, with 0.1% agar concentration. Anti-serum was applied in a central well and homologous or heterologous antigens in the peripheral wells. Samples of complex medium freshly prepared and without inoculation of protozoa were also tested. Diffusion was carried out at room temperature for 72 hours, followed by washing in 0.9% NaCl solution for 24 hours. After drying, the gels were stained with acid fuchsin.

**Biological protection tests** — Two groups of 10 albino male mice (15-20g) were used, one as control and the other as the test group. The solution containing different concentrations of the purified exoantigen (without Freund's adjuvant) was injected subcutaneously in mice of the test group at seven days intervals during three weeks. They received 0.1 ml volume containing 16mg in the first, 50mg in the second and 20mg in the last injection. Mice of the control group received physiological solution at the same intervals. Seven days after the last injection, the mice of the two groups were challenged intraperitoneally with 50,000 bloodstream forms of *T. cruzi* (CL strain). Trypanosoma count was made

by the method described by BRENER<sup>2</sup> and the mortality evaluation was carried out for 3 months.

## RESULTS

After 48 hours incubation of *H. samuelpessoai* in the complex medium using a controlled environment incubator shaker, the yield was about 1.5 to 2.0g (wet weight) cells in 500 ml. This medium free from microorganism after centrifugation at 10,000g for 40 minutes was submitted to salt fractionation. In order to test the best salt concentration, it was precipitated in a pilot experiment, with solid ammonium sulfate in 0-60% and 60-90% fractions. After exhaustive dialysis and lyophilizations, both fractions showed a positive reaction in the immunodiffusion test but in the polyacrylamide gel electrophoresis the active fraction was predominant in the material precipitated with the higher salt concentration. We decided then to use 50-90% saturation to get the exoantigen crude extract.

Figure 1 shows the electrophoresis pattern of: 1) the 50-90% precipitate from the medium without microorganism growth; 2) the crude exoantigen extract obtained by 50-90% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> saturation and 3) the somatic extract obtained by the same procedure as above from the supernatant of the sonicated *H. samuelpessoai* cells. The exoantigen which corresponds to the stronger band has a mobility similar to one of the components from the somatic crude extract.

Figure 2 shows the result of immunodiffusion tests with *T. hastatus hastatus* immune serum. This *T. cruzi* - like microorganism whose characteristics are being studied in our laboratory shows many similarities to the behavior of *T. cruzi*, but it is not pathogenic to laboratory animals. The immune sera obtained with *T. cruzi* and *T. hastatus hastatus* showed the same pattern of precipitation bands when reacted with *H. samuelpessoai* exoantigen.

Gel filtration of crude extract in Sephadex G-75 separated the exoantigen fraction from brownish inactive material. The immunologically active fraction (the first peak material) was again gel filtered in Sephadex G-150; its elution pattern and electrophoretic behavior are shown in Fig. 3 and 4. The exoantigen was present in the last peak (see Fig. 3). The biological tests

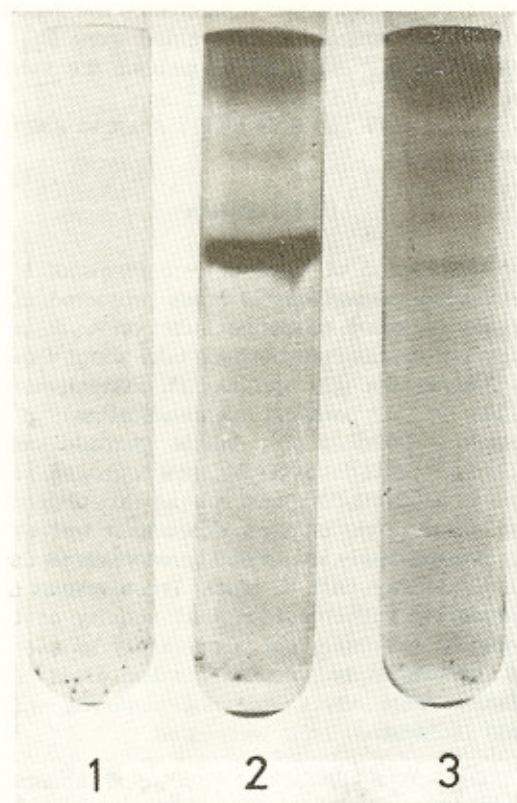


Fig. 1 — Polyacrylamide gel electrophoresis patterns on 7.5% polyacrylamide gel of the fraction salted out at 50-90% ammonium sulfate of the following materials: (1) complex medium; (2) complex medium after *H. samuelpeessoai* incubation; (3) somatic extract.



Fig. 2 — Two tests of the cross reaction between the antigens of *H. samuelpeessoai* and *T. hastatus* immune serum. The central well, in each case, contains *T. hastatus* immune serum. In the right and left peripheral wells were applied crude extract of exoantigen (50-90% salted out) and in the upper and lower wells, crude extract from *H. samuelpeessoai* cells.

were carried out with exoantigen as shown in Fig. 4 containing a slight impurity. However, for the characterization experiments of the exoantigen, the sample was chromatographed

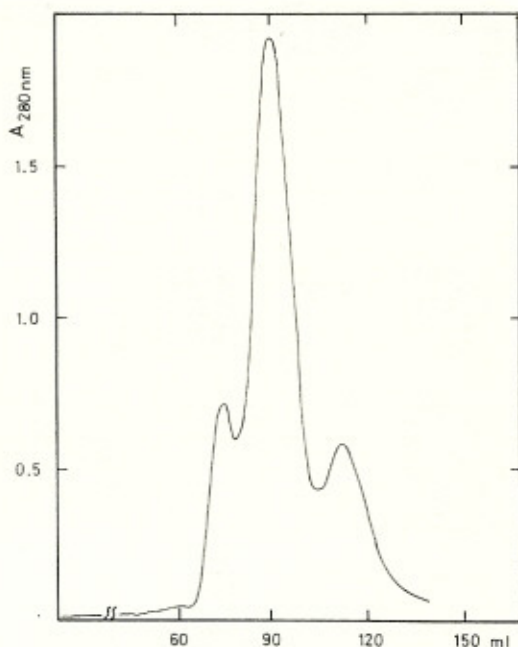


Fig. 3 — Rechromatography of the immunologically active fraction on Sephadex G-150. Sample: first peak material from Sephadex G-75 column. 300 mg of lyophilized material dissolved in 2 ml of 0.05 M phosphate buffer containing 0.2 M KCl and 0.02% sodium azide, pH 7.0. Flow rate: 15 ml/hr; fractions: 3 ml/tube.

once more on Sephadex G-150 column to remove this impurity. Figure 5 shows the results of the protection test in which mice previously immunized with *H. samuelpeessoai* exoantigen received a challenge dose of *T. cruzi* (CL strain).

Parasitaemia levels and mortality index were different in control and immunized groups. In the immunized group the parasitaemia is about 50% less than in the control group and this condition is maintained for at least 3 months.

Some other characteristics of the exoantigen molecule were analyzed. It was shown to be a single protein; the glycoprotein character was not verified in the polyacrylamide gel electrophoresis stained with Alcian Blue. The proteolytic activity was not detected by caseinolytic method of KUNITZ using heat denatured casein as substrate. The isoelectric point was pH 4.2, determined by isoelectric focusing of 400 µg protein samples in polyacrylamide gel (Fig. 6). Through the hydrodynamic parameters, we obtained a value of 58,800 dalton molecular weight for the exoantigen (Fig. 7). On the basis of this molecular weight value and

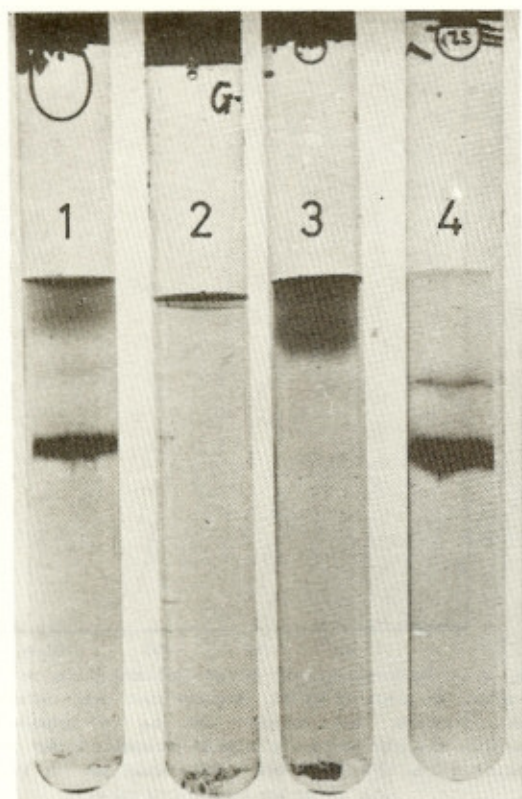


Fig. 4 — Polyacrylamide gel electrophoresis pattern of the fractions obtained in Sephadex G-150 filtration. (1) material before chromatography; (2) first peak fraction; (3) second peak fraction; (4) third peak (exoantigen) fraction.

the U.V. absorption spectrum, the molar extinction coefficients determined were  $E_{278} = 39,900$ , and  $E_{280} = 39,390$ , as well as the values 1% of  $A_{1cm} = 6.77$  and  $6.69$  at 278 nm and 280 nm respectively.

## DISCUSSION

Inoculation of living culture forms of *Herpetomonas samuelpessoai* cross protected mice against *T. cruzi* challenge infection<sup>16</sup>. It is a known fact that macrophages take about 4 days to destroy the incorporated *H. samuelpessoai* (Silva, W. D., personal communication); it is therefore possible that viable parasite cells, although interiorized by macrophages, are able to biosynthesize and release exoantigen in the serum. The isolated exoantigen tested in the present study led to 80% protection 60 days after challenge with *T. cruzi*. These results are compatible with the idea that activity of the living *H. samuelpessoai* is necessary to release the exoantigen responsible for the cross protection. This effect was not observed when dead (sonicated) cells were used.

The cross protection was not observed when mice were injected with the proteins which make up the complex medium. The LIT medium used to maintain the *H. samuelpessoai* inoculum was also tested. The protein of the

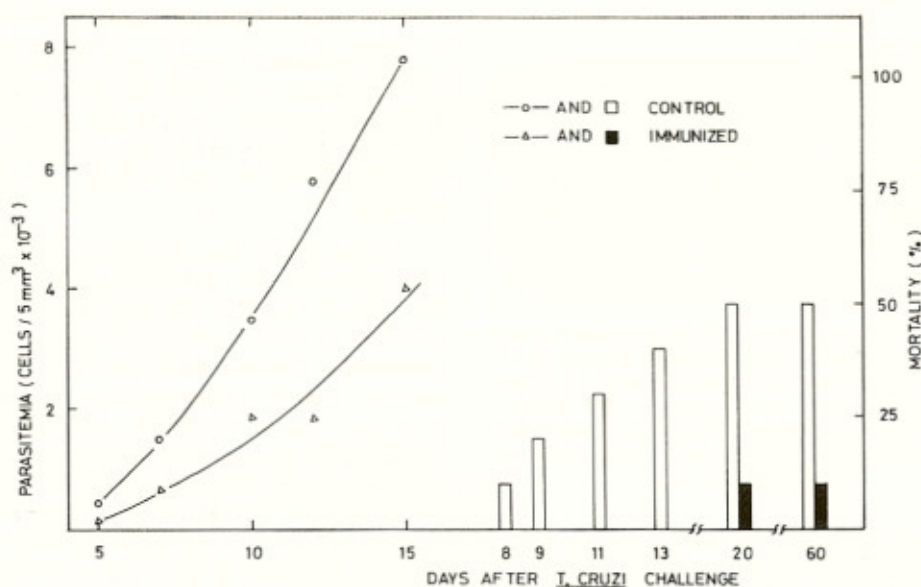


Fig. 5 — Parasitaemia and mortality index in mice immunized with *H. samuelpessoai* exoantigen and challenged with *T. cruzi*.

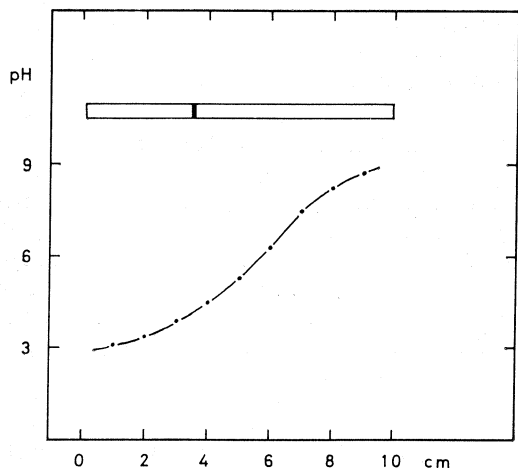


Fig. 6 — Determination of the isoelectric point of *H. samuelpessoai* exoantigen by polyacrylamide gel electrofocusing. The experimental conditions are described in METHODS.

same electrophoretic mobility as the antigen detected in LIT medium without inoculation of *H. samuelpessoai* was isolated by the same procedures and inoculated in mice to analyze the protection effect against *T. cruzi* (CL strain) challenge. All animals died within 30 days after injection. In view of these results we concluded that the protection is not related to any fraction of the medium.

One of the components from the somatic crude extract of *H. samuelpessoai* has an electrophoretic mobility similar to the stronger band corresponding to exoantigen (Fig. 1). Probably because of the very low proportion of this protein in the somatic extract, the cross reaction was not detected in the immunodiffusion test. To clarify this point it is necessary to get a sufficient quantity of the purified somatic fractions, which will be done in the future.

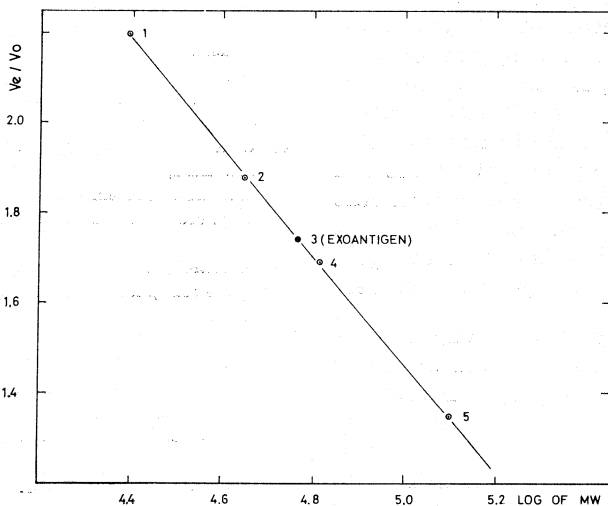


Fig. 7 — Estimation of the molecular weight of *H. samuelpessoai* exoantigen. The Sephadex G-150 column was equilibrated with 0.05 M phosphate buffer containing 0.2 M KCl and 0.02% sodium azide, pH 7.0. Flow rate: 15 ml/hr; (—●—) exoantigen; (—○—) reference proteins.

WEITZ<sup>20,21</sup> described a soluble exoantigen in the serum of rats infected with *T. brucei* which protected animals against homologous infection. TARRANT et al.<sup>18</sup> obtained *T. cruzi* exoantigens *in vitro* which were better than somatic antigen when tested by complement fixation activity. Recently GOTTLIEB<sup>9</sup> detected a polysaccharide originating from *T. cruzi* in plasma of mice acutely infected with this parasite. SEED<sup>15</sup> separated, by elution, a precipi-

tinogen and a protector factor from *T. rhodensiense*. The *H. samuelpessoai* exoantigen molecule characterized in the present study is apparently not a glycoprotein. This conclusion was obtained only by Alcian Blue staining technique. Other methods will be used to confirm this result since TARRANT<sup>18</sup> found the carbohydrate moiety in the *T. cruzi* exoantigen in a ratio 1:1.5 to 1:2.0 between carbohydrate and protein.

Exoantigen from other genera have also been described. SCHNUR<sup>13</sup> demonstrated antigenic components released by *Leishmania tropica* and *Leishmania donovani* which can be used for a differential diagnosis of leishmaniasis by intradermic test. Recently, SCHNUR<sup>14</sup> extended these observations to other genera such as *Leptomonas*, *Crithidias* and *Herpetomonas*.

An experiment to characterize the exoantigen is being carried out in our laboratory. It consists of the incubation of *H. samuelpessoai* in the medium containing radioactive amino acids. There is evidence of the radioactive element being incorporated into the exoantigen molecule. These results, if confirmed, will definitively show that the exoantigen is biosynthesized by cells.

It is a commonly recognized fact that some exoproteins have proteolytic activity; this property, however, was not detected in the *H. samuelpessoai* antigen. The apparently high value of molecular weight confirmed the fact that the dialysis water of exoantigen solution was negative in the immunodiffusion test. The low ratio (1.21) of absorption 278 nm:255 nm indicates that the molecule is poor in aromatic chromophore residues. On the other hand, the maximum at 278 nm means that this protein has more tyrosine than tryptophan residues.

The literature is practically virgin with respect to size and conformation of antigenic macromolecules. Considering that many biological events occur at the level of molecular interactions, more attention should be dedicated to these studies since they could contribute to the elucidation of Chagas' disease immune mechanisms.

## RESUMO

### Exoantígeno de *Herpetomonas samuelpessoai* que protege camundongos contra infecção de *Trypanosoma cruzi*

O presente trabalho descreve a extração, purificação e caracterização de um exoantígeno de *Herpetomonas samuelpessoai* obtido do meio complexo após 48 horas de crescimento a 28°C. Trata-se de uma proteína de peso molecular 58.800 daltons, ponto isoelétrico em pH 4,2, sem atividade proteolítica e o valor de  $E_{280nm} = 39.900$ . Os camundongos imunizados com este

exoantígeno mostraram uma considerável proteção contra uma dose maciça de *Trypanosoma cruzi* injetada intraperitonealmente.

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