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SEROLOGICAL, ELECTROPHORETIC AND BIOLOGICAL PROPERTIES OF Fasciola hepatica ANTIGENS

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SUMMARY

Fasciola hepatica somatic antigen, its partially purified fractions and excretion-secretion products were investigated as to serological, electrophoretic and biological properties. In a Sephadex G-100 column (SG-100), **Fasciola hepatica** total antigen (FhTA) gave 5 fractions, and SDS-PAGE analysis showed they were glycoproteins ranging from 14 to 94 kDa molecular weight (MW). When these fractions were analyzed by enzyme linked immunotransfer blot (EITB) and immunodiffusion in gel (ID) with serum from immunized rats with FhTA, the presence of different antigenic components was revealed.

In the SDS-PAGE of excretor-secretor antigen (ESA), it was possible to observe peptides from 12 to 22 kDa, which were also present in FhTA. When the FhTA, its fractions and the ESA were analyzed by EITB with the immune rat serum (IRS), it was observed that only some fractions of the SG-100 shared antigens with the FhTA and ESA.

Moreover, DTH and ITH responses were studied in FhTA immunized rats challenged with these different antigen components, revealing that the protein/carbohydrate ratio is important for inducing DTH response. The ESA was the most active component in the DTH and ITH response.

KEY WORDS: Fasciola hepatica; Immune response; Excretor-secretor antigen; Antigens characterization.

INTRODUCTION

Fasciola hepatica is a fluke of complex evolutive cycle in which man as well as bovine, ovine and caprine cattle are its definitive hosts, producing biliary obstruction and fibrosis, with parenchyma atrophy and periportal cirrosis. Numerous studies have evaluated different antigen preparations in a diversity of tests for immunodiagnosis of **Fasciola hepatica** infection. Persistent problems of non specificity of tests have encouraged investigation toward the isolation of purified and species-specific antigens for the immunodiagnostic purposes[12].

One of the main limitations for the study of specific immune response has been the lack of well defined parasite antigens. In order to examine the association between the antigen structure and the engendred immune response, it requires to characterize biochemically the antigens that could stimulate different branches of the immune system.

The biological function of the released antigens by parasites is unknown but, it is speculated that they could play a significant role in the host-parasite relationship for they can act at distance[5]. There is evidence that the excretor-secretor antigens are implied in the host immune mechanisms in some nematode infections[14]. The aim of this study was to further characterize a **Fasciola hepatica** total antigen (FhTA) in comparision with its fractions obtained by filtration in Sephadex G-100 (SG-100) columns, as well as with the parasite excretor-secretor products, by Ouchterlony double immuno diffusion (ID) in a gel, polyacrylamide gel electrophoresis (PAGE), and enzyme-linked immunotransfer blot (EITB).

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In addition, the ability of parasite component(s) to elicit cell-mediated immunity (CMI) was assessed by delayed type hypersensitivity (DTH) and immediate type hypersensitivity (ITH) responses in rats sensitized with FhTA.

MATERIALS AND METHODS

Antigen preparation

Fasciola hepatica total antigen (FhTA):

Adult **F. hepatica** worms were obtained from infected bovine livers, washed in 0.01 M phosphate buffered saline, pH 7.2 (PBS), at room, temperature and homogeneized by mechanical disruption in tissue grinder containing 0.8mM phenyl methylsulfonylfluoride (PMSF). The homogenate was then sonicated for 1 minute, centrifuged at 10.000 g at 4°C, for 1 hr. The supernatant obtained was fractionated and stored at -20°C until used.

Fasciola hepatica excretory-secretory antigens (ESA):

Live and intact adult **Fasciola hepatica** worms were obtained from bovine livers at local abattoir and washed 3-4 times with PBS at room temperature during 1 hr, to remove all the blood and bile traces. The worms were then incubated (40 worms/ 100 ml) in PBS at 37°C for 3 hr. The supernatant was centrifuged at 10.000 g at 4°C for 1 hr to remove particulate material. After centrifuging, the volume was reduced 10 times in Sephadex.

Gel filtration chromatography

Fasciola hepatica total antigen was fractionated using a 1.1 x 80 cm. glass column containing SG-100 equilibrated in PBS. Thirty six mg protein were applied to the column. Fractionation was done by using a peristaltic pump, with PBS as the eluant at a flow rate of 14 ml/hr. The collected fractions of 2.5 ml each were analyzed for absorbance at 280 nm, in a spectrophotometer.

The reactivity of the fractions obtained by filtration in the SG-100 column was assessed by counterimmunoelectrophoresis (CEP)[2] using concanavalin A.

Gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to LAEMMLI[7] in 10% polyacrylamide gel slabs in Tris-glycine buffer, pH 8.3. The FhTA and ESA were dissolved in a sample buffer (pH 6.8) consisting of 31.25 mM Tris base 1% SDS, 2% 2-mercaptoethanol and 5% glycerol. In all experiments the stacking gel consisted of 5% acrylamide prepared in 125 mM Tris-HCl buffer (pH 8.6) containing 1% SDS. All reagents were commercially supplied (Sigma Chemical Co.). To estimate the MW of the separated proteins two calibration kits were employed (MW-SDS-200 and MW-SDS-100 B). The gels were fixed and stained for carbohydrates and proteins with PAS[17] and Coomassie blue [11], respectively.

Concanavalin A affinity chromatography

The fraction I of SG-100 (4 mg/ml 0.05 M Tris HCl, pH 7.2) was applied to a column (0.5 x 6 cm) of Con A-Sepharose 4B (Sigma Chemical Co.). The column was successively eluted with: 1) 0.5M NaCl in 0.01M Tris buffer, pH 7.2, containing $MnCl_2$ (1mM) and $CaCl_2$ (1mM); and 2) 0.8 M D-glucose. The elution was monitored at 280 nm using a spectrophotometer [16]. The two fractions obtained by Con A affinity Chromatography were designated as: non-binding Con A (NB-Con A) and binding Con A (B-Con A).

Protein and carbohydrate determination

The protein and carbohydrate contents of the parasite antigenic components were determined acording to LOWRY et al[9]and the phenol-sulfuric acid method[3], respectively.

Enzyme-linked immunotransfer blot (EITB)

SDS-PAGE was carried out on slabs according to LAEMMLI [7]. Gels were comprised of a 5% acrylamide stacking gel and a homogenous 10% acrylamide separating gel. The proteins separated by SDS-PAGE were electrically transferred to nitrocellulose[15]. The nitrocellulose sheets were then incubated with immune rat serum (IRS) at 1/5 dilution in the 50 mM Tris-HCl, pH 7.4, containing 5 mM EDTA; 150 mM NaCl, 0.25 % gelatine and 0.1% Triton x-100 (BBB) buffer at 4°C for 24 hr. Fixed IgG was revealed with peroxidase-protein A conjugate (Sigma Chem. Co.), followed with 3 mg of 4-Cl-naphtol previously dissolved in 1 ml methanol added to 3 ml 50 mM Tris-HCl buffer (pH 7.4) containing 3 µl H_2O_2.

Immune rat serum (IRS)

Inbred white rats of both sexes, 8-12 weeks of age, were immunized subcutaneously with 1 ml emulsion of FhTA (2.5 mg/ml) in complete Freund's adjuvant (CFA) (1:1 V/V ratio). The IRS was obtained at 35th day post immunization (p.i.).

Double immunodiffusion (ID)

Double immunodiffusion gels were prepared on slides, using 1% agarose in PBS[10]. The FhTA was used at 2 mg/ml protein concentration, while the fractions and the IRS were not diluted. As controls, normal rats sera (NRS) were used.

Moreover, liver homogenate, bile, and normal bovine serum were used as control of IRS. The immunodiffusion slides were incubated in moist chambers for 48hr, before the assessment of precipitation lines.

Skin testing in rats

Twenty one days p.i. with FhTA-CFA, the rats were challenged into the right rear footpad with 0.1 ml (1mg/ml) of FhTA or different antigenic fractions, as well as with ESA. As control, an equal volume of 0.15 M NaCl was inoculated, into the left rear footpad. The right and left pads were measured before injecting the antigen or saline as well as 20 min and 48hr thereafter. The rat footpad increase in thickness was determined as follows:

$$(A-B) \times 100 = C$$

A: footpad diameter (mm) 20 min or 48 hr after the injection.
B: footpad diameter (mm) before the injection.

A value of C equal or higher than 30 was considered positive for footpad test.

DTH and ITH negative controls in these studies were normal rats only challenged with the appropriate antigen preparation.

Statistical analyses

Means, standard errors of the means, and unpaired Student's tests were used to analyze the data.

RESULTS

Electophoresis in polyacrylamide gel (SDS-PAGE).

The FhTA, the 6 fractions obtained by filtration in the SG-100 column (Fig.1) and the ESA were analyzed by 10% SDS-PAGE. Protein and carbohydrate concentrations are presented in the Table 1.

The electrophoretic profile of FhTA was characterized by several bands ranging from 14 to 94 kDa, by staining with Coomassie blue (Fig. 2A) and PAS (Fig. 2B).

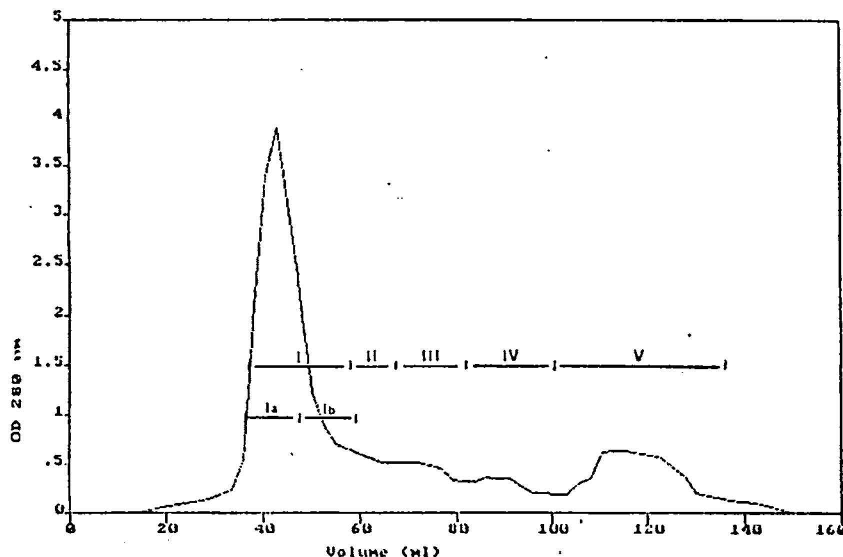

Fig. 1. Elution Profile of FhTA fractioned by Sephadex G-100. Five fractions were separated: I (Ia and Ib), II, III, IV and V.

Table 1
Protein and carbohydrate contents of FhTA, SG-100 fractions and ESA

	Proteins★ (mg/ml)	Carbohydrates# (mg/ml)
FhTA	9.00	4.73
Ia	1.00	0.87
Ib	1.84	0.13
I	1.60	0.46
II	4.50	1.25
III	0.29	2.43
IV	1.08	0.12
V	0.12	0.09
ESA	1.00	4.45

★ Determined by Lowry method.
Determined by phenol sulfuric acid method.

The Ia and Ib fractions showed identical electrophoretic profiles (Fig. 2A and 2B) and both reacted serologically in the similar way as assayed with the IRS (fig. 5A). So, they were joined as fraction I. In SDS-PAGE, the fraction I (stained with Coomassie blue) showed several bands with MW ranging from 14 to 43 kDa, with predominant polypeptides of 19.5 and 43 kDa. Fraction II contained 2 main components of 19.5 and 52.5 kDa, while fractions III and IV contained mainly polypeptides of 18 and 19 kDa. In these two latter fractions, differences in their chemical composition were seen by the sodium periodate treatment and silver staining of the SDS-PAGE gels (unpublished data).

In the stained gels with PAS, only a band of 43 kDa was identified in fraction I, while in fraction II, the components of 19.5 and 52.5 kDa were seen and others of intermediate MW of lower were weakly stained. In fractions III and IV, only a band of fast migration of approximately 18 kDa was observed. The absence of Coomassie blue and PAS stained bands in fraction V might be due to low protein and carbohydrate concentrations.

The SDS-PAGE analysis of the ESA revealed the presence of at least 4 diffuse bands ranging from 12 to 22 kDa after Coomassie blue staining (Fig.3), while only a band of low MW (18 kDa) was revealed with PAS staining.

Concanavalin A affinity chromatography

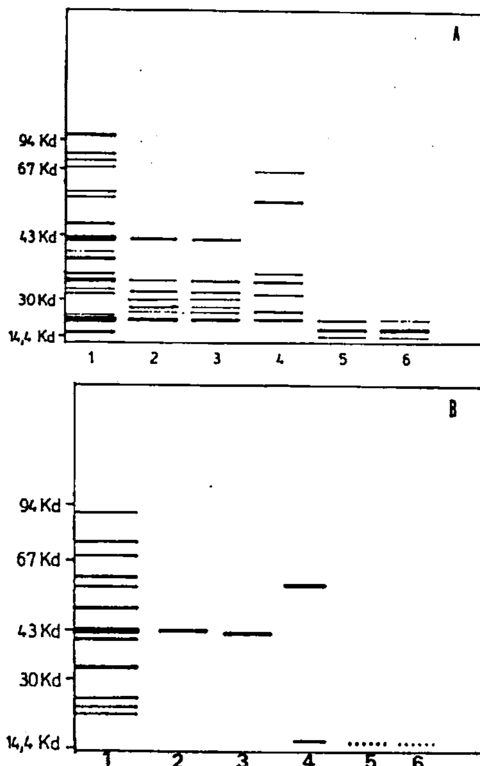

Fig. 2. Coomassie blue-stained polyacrylamide gel (A) and PAS-stained gel (B) of FhTA (lane 1), Ia fraction (lane 2), Ib fraction (lane 3), II fraction (lane 4), III fraction (lane 5), IV fraction (lane 6). Molecular weights expressed in kilodaltons.

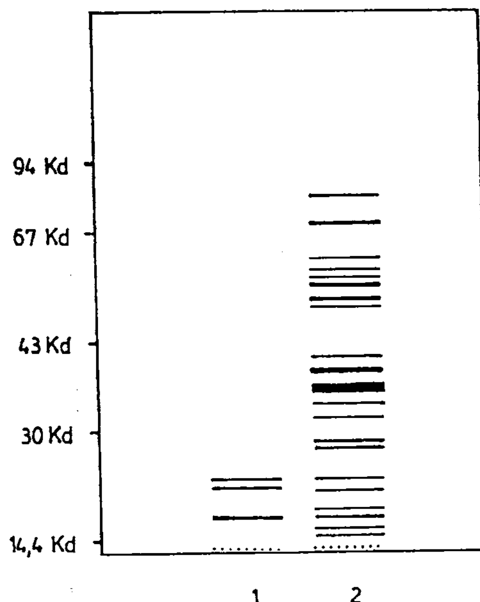

Fig. 3. SDS-Page of ESA (lane 1) and FhTA (lane 2). The gel stained with Coomassie blue.

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The glycoproteic nature of the FhTA was proved by CEP, reacting the fractions obtained from the SG-100 with Con A. The FhTA and fraction I displayed positive reaction with Con A resulting in 2 precipitation bands. Thus, the fraction I was separated in a Con A-Sepharose column.

Two peaks were obtained: peak I did not link to Con A (NB-Con A), and was recovered in buffer Tris-HC1, and peak II linked to Con A (B-Con A) and was eluted with buffer a-D-glucose 0.8 M (Fig.4).

Serologic evaluation

The FhTA and its fractions were evaluated by ID with IRS. As shown in Fig. 5 A, the FhTA presents at least 2 precipitation lines against the IRS (well 1). Fractions Ia and Ib (wells 2 and 3) reacted with IRS giving 3 precipitation lines which showed identity with those of FhTA.

Fractions II, III and IV (wells 4, 5 and 6) revealed one precipitation line each, displaying also identity with the FhTA. However, the fraction III had partial identity with the fraction IV.

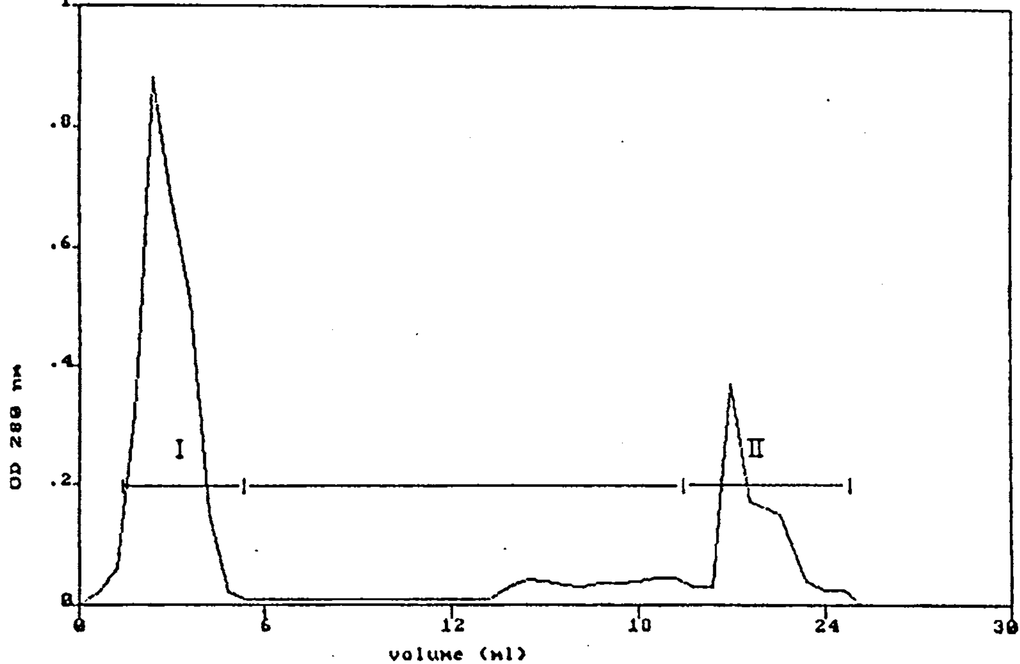

Fig. 4. Concanavalin A affinity chromatography of fraction I from SG-100. The column successively eluted with: 1, 0.5M NaCl-0.01 M tris buffer, containing $MnCl_2$ (1 mM) and $CaCl_2$(1 mM) (Peak I NB-CON A); and 2, D-Glucose (0.8 M) (Peak II B-CON A). The elution monitored continuously at 280 nm.

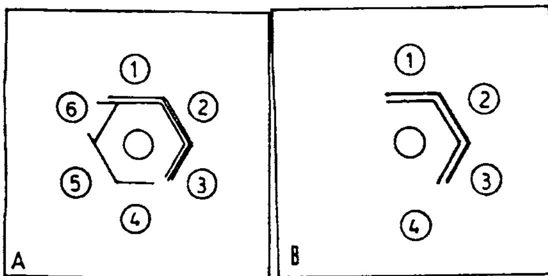

Fig. 5. Double ID gels showing precipitation reactions. (A) anti FhTA rat serum (central well), FhTA (well 1), fraction Ia (well 2), fraction Ib (well 3), fraction II (well 4), fraction III (well 5) and fraction IV (well 6). (B) IRS (central well), FhTA (well 1), fraction I of SG-100 (well 2), NB-CON A (well 3) and B-CON A (well 4).

The fraction V had no precipitation line with the antiserum. The NRS gave no precipitation line with the FhTA; nor IRS precipitated when assayed with liver homogenate, bile and normal bovine serum.

When the peaks obtained by affinity chromatography and fraction I of the SG-100 were tested against IRS, peak 1 (NB-con A) gave 2 precipitation lines, revealing immunological identity with fraction I of the SG-100, while peak II had non-antigenic activity (Fig. 5 B). Thus, the FhTA com-

ponents rich in α-D-glucose and α-D-mannose were non-antigenic by this methodology.

Enzyme-linked immunotransfer blot

The FhTA was evaluated by immunoblotting technique with the IRS and, at least, 4 bands of high MW ranging from 98 to 240 kDa and one of low MW of 21 kDa could be demonstrated. As to the immunoblotting of fraction I of the SG-100, 7 polypeptides of 21, 23, 56, 64, 125, 145, and 160 kDa MW were observed. In fraction II polypeptides of 88, 92 and 125 kDa were detected and in both fractions III and IV one peptide of 88 kDa. The IRS recognized polypeptides in the ESA of 21, 115 and 180 kDa MW (Fig.6).

Elicitation of DTH and ITH responses

To discern which of the antigenic components might be responsible for the DTH response, the rats immunized with FhTA-CFA, at 21° day p.i. (the day of maximum response previously determined in a DTH kinetic experiment), were challenged with FhTA, the fractions and ESA. Groups of normal rats challenged with different antigenic components were also included in this study as controls.

Positive DTH response with the FhTA and the ESA was observed in comparison with its controls ($p<0.003$ and $p<0.001$ respectively).

Among different fractions, the only one that did not give a positive response was the fraction V (Fig.7). Although fractions B-Con A and NB-Con A gave positive responses, no statistically significant difference was observed between both fractions (data not shown).

The DTH responses to the different fractions and to the ESA were compared with the response to the FhTA, and the responses provided by frac-

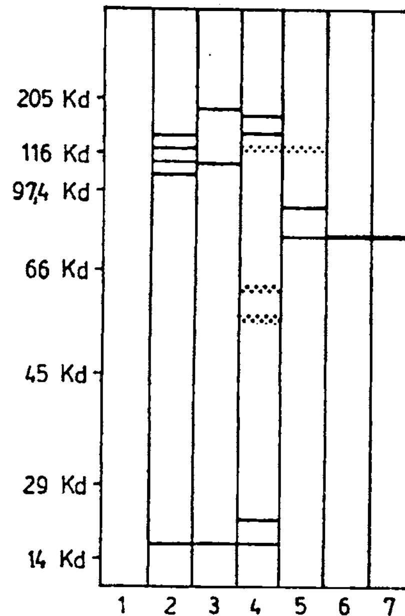

Fig. 6. Immunoblots of SDS-PAGE of antigen preparations. FhTA(lines 1 and 2), ESA (line 3), fractions I, II, III and IV obtained by SG-100 (lines 4, 5, 6 and 7) respectively. Line 1 probed with nrs and the others with irs. Molecular weights are expressed in kilodalton.

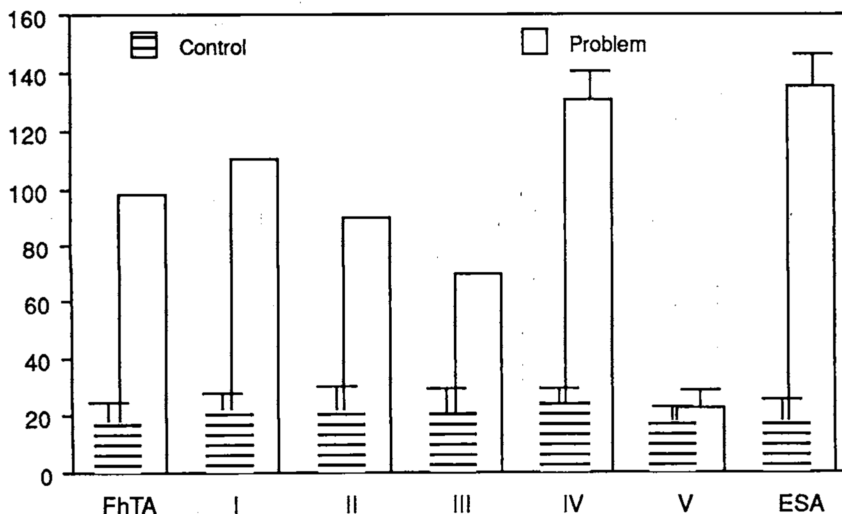

Fig. 7. DTH reactions of untreated rats (negative controls) (▤), and rats immunized with FhTA in CFA (□), after footpad challenge with: FhTA; fractions: I; II; III; IV; V; and ESA.

tion IV ($p<0.03$) and by ESA ($p<0.02$) were demonstrated to be better. Unlikely, fractions I, II, and III (Fig.7) did not give statistically significative responses.

The same antigenic components used in the DTH response were also studied for ITH reaction in rats immunized with FhTA-CFA on the day 35 p.i. (the day of maximum responses previously determined). It was observed that the FhTA and fractions III and IV gave a similar positive responses when compared with their respective controls ($p<0.004$). Fractions I and II (Fig.8) gave also positive responses, in regard of their controls, although they were significantly inferior to that obtained with the FhTA ($p<0.005$ and $p<0.003$, respectively). Fraction V did not give positive response in relation to the control (p=NS). The highest value was obtained in ITH with ESA in relation to its control ($p<0.0001$) and to FhTA ($p<0.005$).

DISCUSSION

Previous studies showed that FhTA specifically detects CMI responses in immunized animals[1]. However, FhTA is not a homogeneous material since it comprises at least 30 and possibly more constituents, as demonstrated by the electrophoretic patterns after PAS and Coomassie blue stainings in this study.

SDS - PAGE analysis of ESA evidenced the presence of at least 4 Coomassie blue stained polypeptides and a low MW band revealed by PAS; being all these components also present in the FhTA. In the different fractions of the FhTA, glycoprotein components of middle and low MW ranges are found in fractions I and II, whereas in fractions III and IV, only low MW components were seen.

When these fractions were assessed in the ID test, IRS identified, at least, two antigenic components in the FhTA showing precipitation lines of identity with fraction I, and one of the components with fractions II, III and IV. In the purification of fraction I (by affinity chromatography with Con A), it was observed that the α-D-mannose and α-D-glucose residues are non antigenic components.

Many investigators have reported the isolation of antigenic fractions with specificity for fasciolosis [6,12,13] however, few date are available on antigen fractions which had been characterized and evaluated in respect to their stimulating or non stimulating capacities of cellular and/or humoral immune responses[4].

LEHNER & SEWEL[8], fractioning excretory-secretory antigen by SG-200, have found by ELISA that sera from infected sheep, rats and rabbits reacted mainly with the higher MW bands, although rabbit and sheep sera also reacted with some low MW components. Similar results were published by RIVERA MARRERO et al.[12] in experimentally infected mice, in which antigenic components were identified in all the tested fractions, being those of higher MW (150-160 kDa)

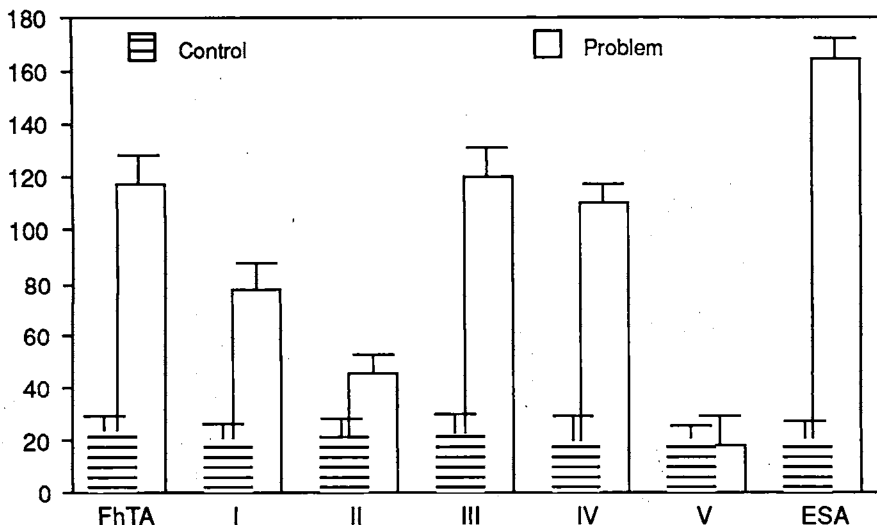

Fig. 8. ITH Reactions of untreated rats (negative controls) (▤), and Rats Immunized with FhTA in CFA (□), after footpad challenge with: FhTA; Fractions: I; II; III; IV; V; and ESA.

associated to the primary fasciolosis. SANTIAGO et al[13] verified that both sheep and cattle sera recognized **Fasciola hepatica** somatic antigens which differed from those prominently detected in the ES product. In the somatic antigens, both sheep and cow sera recognized the major bands of 30 to 38 kDa, whereas in the ES antigen, a group of polypeptides of approximately 23 kDa was recognized.

In the EITB the IRS recognized bands of high MW (115 kDa) in the FhTA and in the ESA, as well as in fractions I and II of the FhTA. Nevertheless low MW (21 kDa) was only found in fraction I. A 88 kDa antigenic component was detected in fractions III and IV. Glycoproteic molecules (18 and 19 kDa) were observed in these two fractions, probably being similar to those described by ESPINO[4] a low MW glycoproteic fraction of **Fasciola hepatica** antigen.

The fractions obtained in the present work were used to determine the cutaneous reactivity test in immunized animals with FhTA. All the antigenic components gave positive DTH responses, and the lower value was obtained with fraction III when compared with that of FhTA. In contrast, the fraction IV showed the highest response ($p<0.03$). The former fraction was seen to be consisted of carbohydrates, whereas the latter comprising mainly proteins. Thus, it could be suggested that the protein/carbohydrate ratio of each fraction may play an important role for the induction of DTH response in the present experimental system. Also, the greatest DTH response was obtained with the ESA which is mainly composed of carbohydrates. These findings would be indicating that besides the protein concentration, the composition epitopes and its ability to induce DTH should be evaluated. In the ITH response, it was observed that fractions III and IV developed a good positive response. Both carbohydrate and protein fractions seem to induce ITH response. Moreover, fraction II had a significantly diminished ITH response in comparison to FhTA ($p<0.002$).

This fraction is consisted of a high concentration of carbohydrates and of proteins. These results allow to deduce that the protein/carbohydrate ratio would not be the main factor in inducing the ITH. On the other hand in both types of cutaneous response, DTH and ITH, the α-D-glucose and α-D mannose residues would not particularly be influencing the response.

The present data provided information to further investigate, in a selective way, the high and low MW antigenic components contained in the ESA which might in association or not be responsible for the CMI and ITH stimulus, in addition to their involvement in the regulation mechanisms of the evasion of the immune response.

RESUMEN

Propiedades serologicas, electroforeticas y biologicas de antigenos de *Fasciola hepatica.*

Se realizó la purificación parcial de un antigeno somático de **Fasciola hepatica** y se obtuveron los productos de excreción-secreción. Por filtración del antigeno total de **Fasciola hepatica** en Sephadex G-100 (SG-100), se obtuvieron 5 fracciones las que al ser analizadas por electroforesis en geles de poliacrilamida, demostraron estar constituidas por glicoproteínas con un rango de peso molecular (PM) entre 14 y 94 kDa. Cuando estas fracciones fueron analizadas por immunoblot e inmunodifusión en geles de agar frente al suero de ratas inmunizadas con el homogenato total se reveló la presencia de diferentes componentes antigénicos.

Usando un antígeno excretor-secretor fue posible observar en el perfil electroforético la presencia de péptidos con un PM entre 12 y 22 kDa, los cuales también estan presentes en el antígeno total. Cuando el antígeno total, sus fracciones y los productos de excreción-secreción fueron analizados por immunoblot frente a suero de rata inmune, se pudo demostrar que algunas fracciones obtenidas por filtración en SG-100 comparten antígenos con el homogenato total y el excretor-secretor.

Por otra parte, cuando se estudio la respuesta de hipersensibilidad demorada e inmediata en ratas inmunizadas con el antígeno total, y desafiadas con los distintos componentes antigénicos se observó que la proporción proteínas/carbohidratos de cada fracción es importante en la inducción de la respuesta inmune celular. El antígeno excretor-secretor demostró ser el componente mas activo en ambos tipos de respuesta.

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REFERENCES

1. CERVI, L.; BORLETTO, N. & MASIH, D.T. - Jornadas de Bioquímica Clínica. ABC. Córdoba, Septiembre 1990.

2. CHERNIAK, R.; REISS, E. & TURNER, S.H. - A galactoxylomannan antígen of **Cryptococcus neoformans** serotype A. **Carbohydr. Res., 103:** 239-250, 1982.

3. DUBOIS, M.A.; GILES, A.K.; HAMILTON, J.K.; REBERS, A. & SMITH, F. - Colorimetric method for determination of sugars and related substances. **Analyt. Chem., 28: 350-356, 1956.**

4. ESPINO, A.M.; PICO, M.C.; LOPES, S.; DUMENIGO, B. E.; BARBAN, D. & HUESCA, N. - Purificación y caracterización parcial de un antígeno somático de **Fasciola hepatica. Rev. cuba. Med. trop., 39:** 23-31, 1987.

5. GAMBLE, H. R. - Comparison of immune effects in mice immunized with **T. spiralis** adult and larval antigens. **J. Parasit.,71:** 680-684, 1985.

6. HYLLIER, G.V. & SANTIAGO DE WELL, N. - Partial purification of **F. hepatica** antigen for the immunodiagnosis of fascioliasis in rats. **J. Parasit, 3:** 430-433, 1977.

7. LAEMMLI, U. K. - Cleavage of structural proteins during the assembly of the head of bacteriophage T4. **Nature, 277:** 680-685, 1970.

8. LEHNER, R. P. & SEWELL, M.M. - A study of the antigens produced by adult **Fasciola hepatica** maintained in vitro. **Paras. Immunol., 2:** 99-109, 1980.

9. LOWRY, O.H.; ROSERBROUGH, H.J.; FARR, A. L. & RANDALL, R. J. - Protein measurement with the folin phenol reagent. **J. biol. Chem., 193:** 265-275, 1951.

10. OUCHTERLONY, O. - Diffusion in gel methods for immunological analysis. **Progr. Allergy, 5:** 1-78, 1958.

11. PIERCE, J. & SUELTER, C. H. - An evaluation of the Coomassie Brilliant Blue G 250 Dye binding method for quantitative protein determination. **Analyt. Biochem., 81:** 478-480, 1977.

12. RIVERA MARRERO, C.A.; SANTIAGO, N. & HILLYER, G. V. - Evaluation of immunodiagnostic antigens in the excretory-secretory products of **Fasciola hepatica. J. Parasit., 74:** 646-652, 1988.

13. SANTIAGO, N.; HILLYER, G.V.; GARCIA-ROSA, M. & MORALES, M.H. - Identification of functional **Fasciola hepatica** antigens in experimental infections in rabbits. **Int. J. Parasit.,14:** 197-206, 1988.

14. SILBERSTEIN, D.S. & DESPOMMIER, D.D. - Effect on **Trichinella spiralis** of responses to purified antigens. **Science, 227:** 948-949, 1985.

15. TOWBIN, H.; STRAEHELIN, T. & GORDON, J. - Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. **Proc. nat. Acad. Sci.** (Wash), **76:** 4350-4354, 1979.

16. TURNER, V. C.; CHERNIAK, R. & REISS, E. - Fractionation and characterization of galactoxylomannan from **Cryptococus neoformans. Carbohydr. Res., 125:** 343-349, 1984.

17. ZACHARIUS, R.M.; ZELL, T.E.; MORRISON, J.H. & WOODLOCK, J.J. - Glycoprotein staining following electrophoresis acrylamide gels. **Analyt. Biochem., 30:** 148-152, 1969.

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