Immunocytochemical localization of antigenic structures of *Trichinella spiralis* larvae by light and electron microscopy

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Summary

A study was realized in order to determine the localization of antigens in the muscular larvae of *Trichinella spiralis*. Antisera obtained form a experimentally infected dog were used. Small pieces of diaphragm from infected mice were embedded in Araldit Luft. Semithin histological sections were incubated with 1/1000, 1/2000 and 1/5000 antisera dilutions and protein A peroxidase resulting a specific reaction on the cuticle, stichocytes and luminal surface of the intestinal cells. The brown staining intensity was dependent on the antisera dilutions. At a subcellular level, ultrathin sections were incubated with the same antisera dilutions and protein A gold conjugate. Specific inmunolabelling complex were present on the globules of the stichocytes and the epicuticle at 1/5000 antisera dilution. Nonspecific reactions were seen when were incubated at 1/2000 antisera dilution. No gold particles were present in the cytoplasm of the nurse cells. The present study has shown that the immuno electron microscopy with colloidal gold is efective for detecting different antigens of *T. spiralis* larvae during natural infections.

Key Words: *Trichinella spiralis*; Immunoelectronmicroscopy; Protein A-gold reaction; Protein A- peroxidase staining; Immunollabeled complex; Immunogenic structures.

Resumen

Se ha realizado un estudio sobre larvas musculares de *Trichinella spiralis*, con el fin de conocer la localización de los antígenos parasitarios, mediante la utilización de antisuero obtenido en perros infectados experimentalmente. Las muestras empleadas procedían de diafragma de ratones infectados, que se incluyeron en Araldit Luft. Cortes semifinos fueron incubados con el antisuero a diluciones 1/1000, 1/2000 y 1/5000 y proteína A peroxidasa, observándose una reacción específica en la cutícula y esticocitos y borde luminal de las células intestinales, cuya intensidad de coloración era dependiente de la dilución del antisuero. Secciones ultrafinas fueron incubadas con el mismo antisuero y mismas diluciones junto a proteína A conjugada con oro coloidal, observándose depósitos de inmunocomplejos específicos en los glóbulos del esticocito y en la epicutícula a dilución 1/5000. Reacciones inespecíficas se encontraron cuando el antisuero era diluido a 1/2000. En ningún caso se observaron partículas de oro en el citoplasma de las células nodrizas. El presente estudio demuestra que la inmunoelectromicroscopia con oro coloidal es efectiva para detectar la localización de los antigenos parasitarios de las larvas de *T. spiralis* durante infecciones naturales.

Palagras clave: *Trichinella spiralis*; Inmunoelectromicroscopia; Reacción proteína A oro; Reacción proteína A peroxidasa; Inmunocomplejos; Estructuras inmunogénicas.

Introduction

One of the main problems to be resolved in parasitic infections is to determine the quality and quantity of each of the antigens produced in the different phases of its life cycle. In order to clarify the situation, a great deal of work has been done over the past years with *T. spiralis*, a parasite whose larvae, enclosed in muscular cells, are easily maintained in experimental animals.

The studies about the function, identification, localization ant elimination of the dif-

ferent antigens have been accomplished using different techniques. Two of the most outstanding being immunolabelling staining (Crandall and Crandall[2]; Despommier and Muller[4]; Pritchard[12]; Mc Laren et al.[8]; Takahashi et al.[18]) and biochemical methodos (Despommier and Laccetti[3]; Parkhouse et al.[11]; Ortega-Pierres et. al.[10]; Gamble et al.[6]). Silberstein[15] pointed out that the principal objetive is to know the role that each antigen plays for the parasite. Therefore, to evaluate this function, certain preliminary information is necessary, such as the location of the antigen in the parasite. The present study was designed to contribute towards this goal by visualizing reactions produced by the larvae in muscle using immunolabelling techniques in preparing the samples for light and electron microscopy.

Materials and Methods

Animals

Mice infected with *Trichinella spirallis* were used. These were sacrificied two months post infection. Samples from their diaphragmatic muscle were fixed for two hours in a solution of glutaraldehyde 1% and paraformaldehyde 2% at 4°C, and washed in cacodylate buffer and dehydrated in a graded series of ethanol solution followed by propylene oxide. Embedding was made in Araldite Luft (Polysciences Inc.). Ultrathin and semithin (1 µm thick) sections were cut for electron and light microscopy in an LKB Nova Ultratome.

Antisera

Two dogs were inmunized with 3000-3500 larvae "per dog" orally administered. The sera were obtained by punction of the safena vein at 52 and 58 days postinfection. Sera were then tested with ELISA, using larval soluble antigens.

Immunoperoxidase in tissue sections

The semithin sections of the samples were desplastificated with sodium methoxide and then rehidrated. The technique was performed according to Sternberger[17], incubated during 48 hours at 4°C, using 1/500, 1/1000, 1/2000 and 1/5000 antisera dilutions in 1% ovalbumin (OVA)-0.05 M Tris buffer saline (TBS) pH 7.6. Normal blood was collected from a dog as a negative control and was used at the same dilutions. The sections were incubated at room temperature with protein A-peroxidase (Sigma) at a 1/100 dilution. Diaminobenzidine tetrahydrochloride (DAB) (Sigma) was used as a chromogen.

Immuno-electron microscopy

Ultrathin sections were cut with a diamond knife and were then collected and placed on nickel grids. The immune labelling procedures were performed according to the proteine A-gold (pAg) technique of Roth[13,14], incubating during 24 hours at 4°C and using 1/200, 1/500, 1/1000, 1/2000 and 1/5000 antisera dilutions in 0.5% OVA-0.05 M TBS pH 7.6. pAg, at 1/100 dilution, with 10 nm sized gold particles (Sigma) was used as anti-IgG. After these were stained with uranyl acetate and lead citrate. All ultrathin sections were examined with a Philips CM 10 electron microscope.

Results

The sections of the muscle stage larvae when incubated with antisera and protein A peroxidae reacted specifically, and the intensity of the reaction was dependent on the dilution of the antisera. Immunostaining complexes were formed in the 1/1000 (Fig. 1E), 1/2000 (Fig. 1C) and 1/5000 (Fig. 1B) dilutions. The only staining by the dark brown peroxidase were located at the cuticle, stichocytes and luminal surface of the intestinal cells of the larvae, and there was no evidence of enzyme deposit on the nurse cells (Fig. 1A). No staining was observed in the negative control using normal dog serum.

At the ultrastructural level, the reactive sites of the larvae coincide with those observed in the peroxidase staining. Using the techniques previously described, it was observed that the distribution of the amounts of

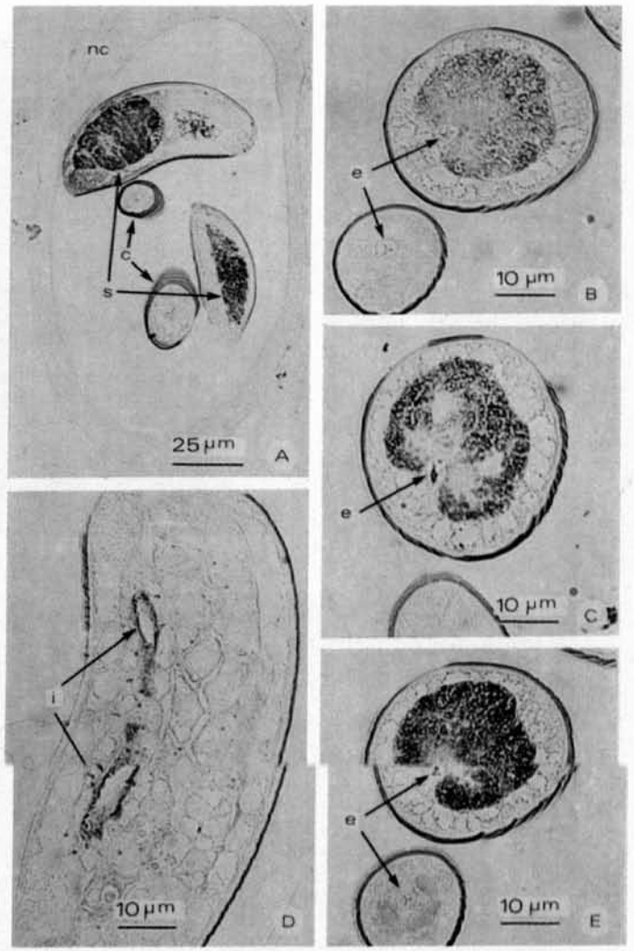

Fig. 1. (A) Semithin section of muscle-stage larvae of *Trichinella spiralis* treated with 1/1000 policlonal antibody obtained from naturally infected dogs followed by protein A-peroxidase. No evidence of enzyme deposits over nurse cell (nc) are shown, the only reaction observed is on the cuticle (c) and stichocyte (s) of the larvae. (B) Cross semithin section of the fore body of the larvae treated with 1/5000 antisera dilution followed by pA-peroxidase. A slight reaction is present in the lumen of the glandular esophagus (e) (bigger section) and a positive inmunostaining reaction on a few globules of the stichocyte. A dark brown peroxidase staining is seen on the cuticle. No staining is observed on the triradiate lumen of the upper esophagus region (smaller section). (C) A section like (B) but treated with 1/2000 antisera dilution. More amounts of DAB deposits than (B) are present in the stichocyte and the posterior region of the esophagus. (D) Oblique semithin section of the hind body of the larvae treated as in (B). Note that the inmunoperoxidase labelling is located on the luminal surface of the intestine (i). (E) A section like (B) but treated with 1/1000 antisera dilution. A strong and uniform reaction is present in all of the section of the stichocyte that surrounds the slightly stained oesophagus. The triradiate lumen of the anterior oesophagus (smaller section) shows no reaction.

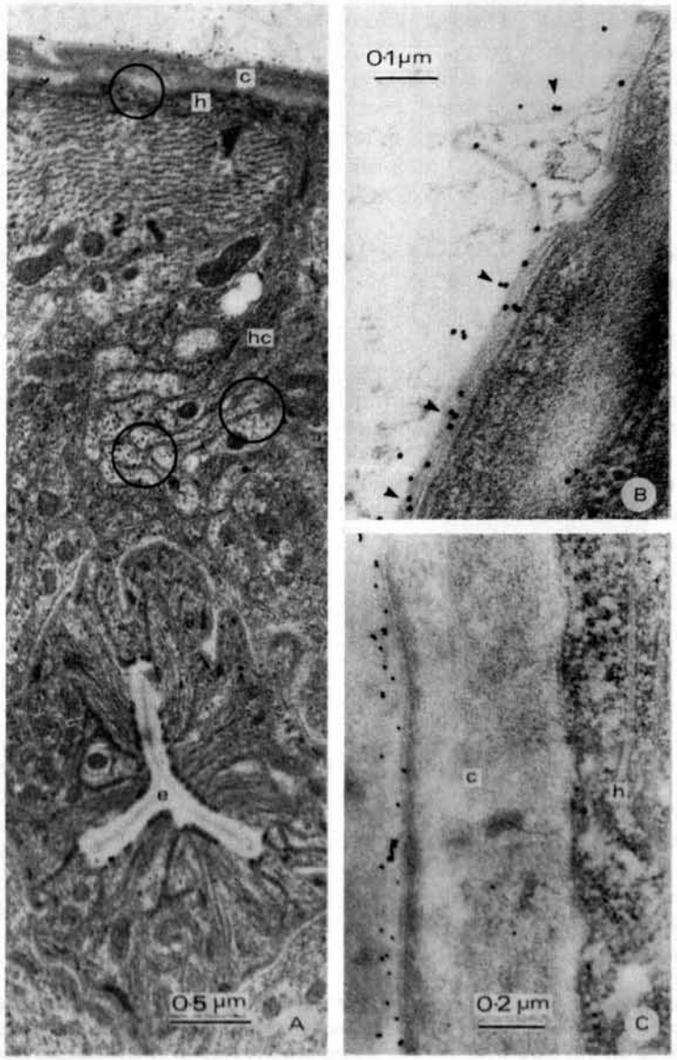

Fig. 2. (A,B,C) Immuno electron microscopy of ultrathin sections of *T. spiralis* larvae incubated with 1/5000 antisera dilution obtained from dog and protein A labelled with colloidal gold of 10 nm. (A) Cross section at the muscular esophagus (e) level. The main concentration of gold particles are present on the surface of the cuticle (c). No specific gold labelling is detected in other structures, however a more or less nonspecific reaction maybe observed on the hypodermis (h) and hypodermal chords (hc) (inside the ring). (B) A high-power magnification of the cuticular region of (A), the gold particles labelling is present over the epicuticle, (head arrows), showing that this is strongly specific to the inmunostaining reactions at this dilution. (C) A section of muscular larvae at the cuticular level. Note that a great deal of gold particles are clustered on the epicuticle. No specific inmunolabelling reactions are seen in other places. (as in the cuticle (c) and hypodermis (h)).

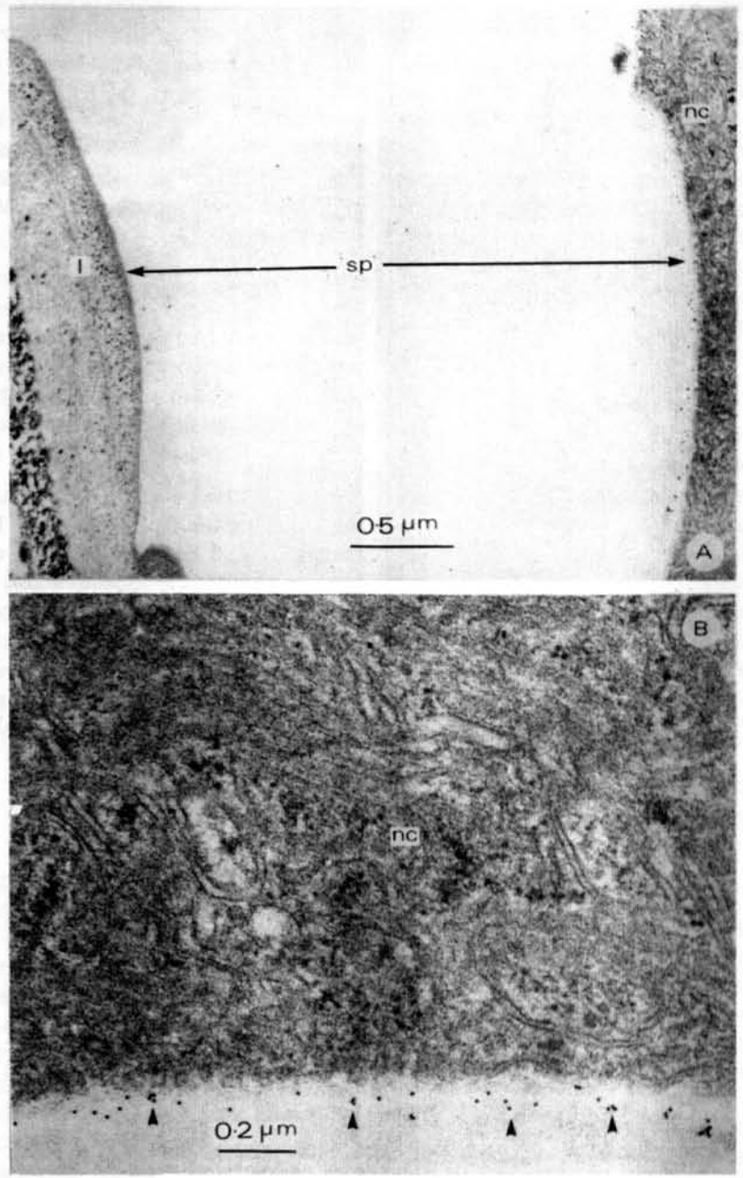

Fig. 3. (A) (B) Inmunmoelectron microscopy of *T. spiralis* larvae treated as in Fig. 2. (A) The space (sp) between the nurse cell (nc) and the larvae (1) can become exagerated. When this occurs, a specific reaction can be present at the border of the nurse cell (as in this case) or over the cuticle (as in Fig. 1B, C). (B) A high-power magnification of the same nurse cell of (A). No gold particles are present in the cytoplasm of the nurse cell (nc). Only positive inmunolabelling complexes can be seen on its border (head arrows).

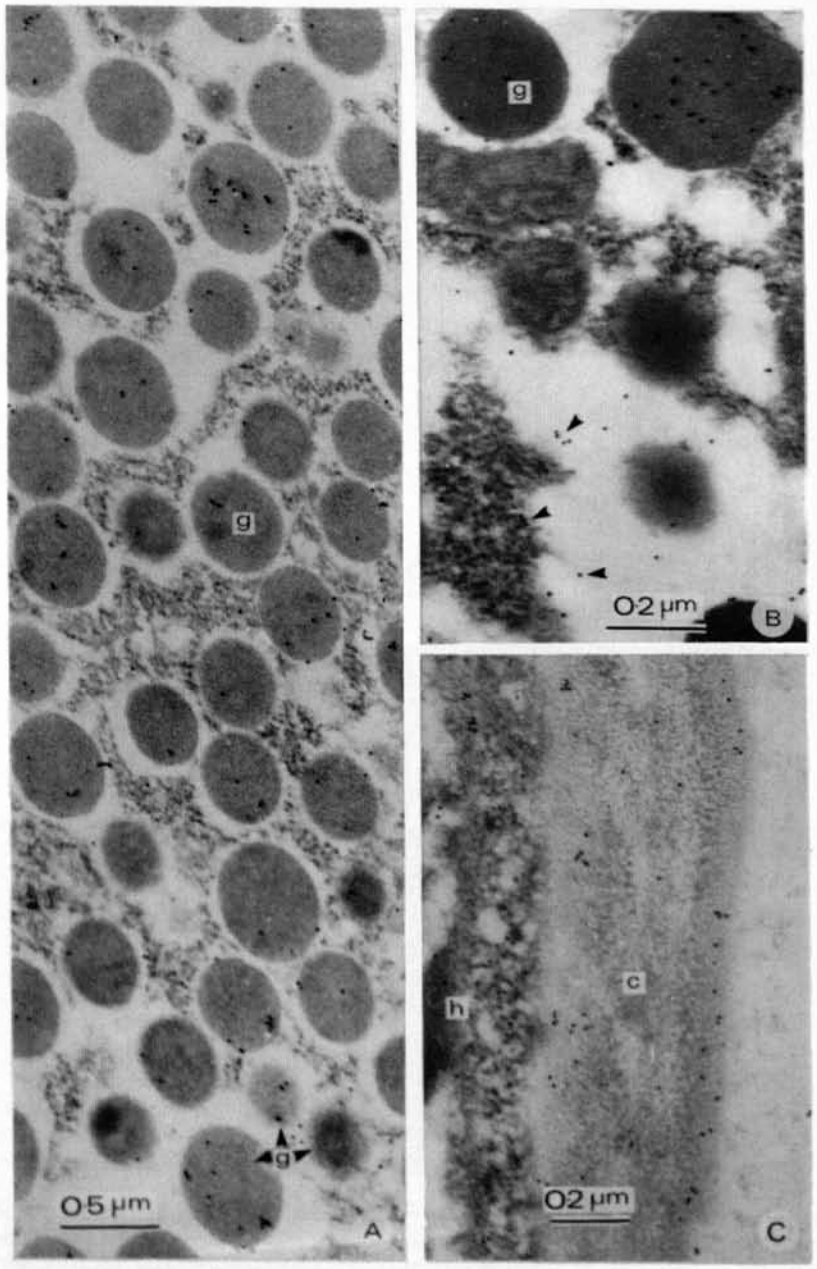

Fig. 4 (A) Specific reaction of the ultrathin section of the stichocyte treated with 1/5000 antisera dilution and pAg. The gold particles are only present over the core of electron dense granules (g) (B,C) Nonspecific reactions of ultrathin sections incubated with 1/2000 antisera dilution and pAg. (B) The gold particles are distributed mainly over dense globules (g) but are also distributed throughout the rest of the cytoplasm (head arrows) (C) Note that the gold particles are scattered all over the cuticle (c) and the hypodermis (h).

gold particles occured at the 1/500, 1/1000 and 1/2000 (Fig. 4B,C) antisera dilutions, while at the 1/5000 dilution a strong specific reaction was detected on the cuticle (Fig. 2A), to be exact at the epicuticle level (Fig. 2B,C) and on the dense globules of the stichocytes (Fig. 4A). A few gold particles were visible on other structures, as in the hypodermis and hypodermal cords, but these reactions were interpreted as nonspecific (Fig. 2A).

As a result of the fixation process one could observe, by electron microscope, that this surface coat was detached from the cuticle and drawn near to the nurse cell. In that case the immunolabelling reaction was only produced on the internal limit of the host cell and not on the cuticle (Fig. 3A).

The reaction that was seen at 1/2000, with gold particles distributed on all stichocytes and cuticle, was interpreted as unspecific (Fig. 4B, C). Only gold particles were detected on the larvae at 1/5000 antisera dilution and they were never found in the cytoplasm of the nurse cell (Fig. 3B). No reaction was observed in the negative control using normal dog serum.

Discussion

Utilizing immunocytochemical techniques distinct immunogenic zons of the muscular larvae of *T. spiralis* have been detected. These zones react with specific anti-*Trichinella* antibodies obtained from different antigenic sources and from diverse animal species. The localization on the parasite of the determinant antigens have been carried out by techniques such as immunofluorescence, immunoperoxidase and others (Despommier and Muller[4]; Pritchard[12]; Kim and Lebdeter[7]).

Regardless of the methods used a strong positive reaction on the cuticle and stichocytes has been observed by Crandall and Crandall[2], Despommier and Muller[4], Pritchard[12] and Mc Laren et al.[8], and on the surface of the intesti- nal cells (Mc Laren et al.[8]) and even in some immunocompetent cells of the wall of the cyst. This indicates that antigens produced during encystment are capable of crossing the cysts boundary and contacting the mononuclears (Pritchard[12]). In all cases the inmunolabelling reaction was present in the stichocytes, the cuticle and the intestine. These results are in accordance with those that we have observed on semithin sections of muscular larvae incubated with specific antibody obtained from orally infected dogs, as if it were a natural infestation, and proteina A peroxidase.

The electron microscope was used in order to obtain more information about the immunological properties of the larvae and adults of *T. spiralis*. Despommier et al.[5] detected immunolabelled complexes on the outer surface of the cuticle using ferritin conjugates. Mc Laren et al.[8] employing pre-embedding labellin procedures by monoclonal antibodies and peroxidase showed a dense enzyme deposit associated with the epicuticle and the intestinal microvilli border, where it seems that the antigens could have pased to the exterior through the intestine and afterwards spread or extended over the cuticle to such extent that, if this surface coat disapears, the reaction does not occur.

Recently Takahashi et al.[18], with the use of proteine A-gold and antisera obtained from rats found immunostaining structures in differents granules of the stichocytes, body cuticle, luminal surface of the intestine, intestinal cells, hypodermis and paraplasmatic inclusion microbodies in hypodermal cords.

Our results are in accordance with the investigators previously cited, although they differ with those of Takahashi et al.[18] in that the distribution of the gold particles were not observed either in the hypodermis hypodermal cords and or at the cuticle level. We believe taht this discrepancy could be due to the fact that they used a different method: a postembedding immunological

staining wiht antisera diluted at 1/200 incubated for 30 minutes at room temperature. It is also possible that there were alterations in the immunogenic components of the cuticle when the larvae were isolated by pepsin digestion, as occured when *Dipetalonema vitae* were treated whit protease digestion by Baschong et al.[1]. The fact that the reaction that we observed was only produced in the epicuticle when this was adjoined to the cuticle or when it was displaced towards the nurse cell, makes one think that the reaction is more specific to enciysted larvae, like the ones we observed, than to the larvae obtained by artificial digestion.

Parkhouse et al.[11] showed that antigens of the cuticle exhibit four labelled proteins of 47, 55, 90 and 105 kDa. Two of this molecules (47 and 90 kDa) have carbohydrate groups which are lectin adherent and are immunogenic during the normal course of infection. Recent studies (Ortega-Pierres et. al.[9]; Mc Laren et al.[8]) have provided evidence that purified surface components are able to induce protection in experimental trichinellosis in mice, manifested by reduced muscle larvae burden annd accelerated expulsion of adult worms.

According to the authors that have been cited previously, the most common and specific reactions, regardless of the methods employed, are those of the stichocytes. Thus, these are the structures which can best be identified with the antigenic activity of the larvae. Silberstein and Despommier[16] identified molecules of 48 and 50-55 kDa, localized by immunocytochemical techniques, from the beta and alpha stichocyte globules respectively, as well as from the cuticle surface. The monoclonal antibodies have been used to localize antigens in sections of the parasite (Silberstein and Despommier[16]; Mc Laren[8]) showing a positive reaction with the surface, stichocyte globules and the border of intestinal cells. In these locations there apperared to be antigens of similar molecular weight or common antigenic determinants. We agree with Mc Laren et al.[8] namely that the antigens are secreted by the stichocytes and after released to the niche throught the mouth and anus. Once eliminated these antigens could cover externally the larvae and then become incorporated into the epicuticle, which in time or by the actions of determined enzymes could change its chemical structure while maintaining intact its antigenic radicals. This fact could explain why antigens of distinct molecular weights and localizations give rise to a identical immunolabelling reaction. The present study has shown that the colloidal gold and the electron microscopy techniques are effective fo recognizing and locating somatic and surface antigens of *T. spiralis* larvae *in vivo*.

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References

1. **Baschong, W.; Tanner, M.; Berschart, B.; Rudin, W.; Weiss, N.** "*Dipetalonema vitae* (Filarioidea): Extraction and inmunogenicity of cuticular antigens from female worms". *Exp. Parasitol.*, 53, 1981, 262-269.
2. **Crandall, R.B.; Crandall, C.A.** "*Trichinella spiralis*: Inmunologic response to infection in mice". *Exp. Parasitol.*, 31, 1972, 378-398.
3. **Despommier, D.D.; Laccetti, A.** "*Trichinella spiralis*: Proteins and antigens isolated from a large-particle fraction derived from the muscle larva". *Exp. Parasitol.*, 51, 1981, 279-295.
4. **Despommier, D.D.; Muller, M.** "The stichosome and its secretion granules in the nature muscle larva of *Trichinella spiralis*". *J. Parasitol.*, 62, 1976, 775-785.

5. **Despommier, D.D.; Kajima, M.; Wostman, S.** "Ferritin-conjugated antibody studies on the larva of *Trichinella spiralis*". *J. of Parasitol.*, 53, 1967, 618-624.
6. **Gamble, H.R.; Rapic, D.; Marinculic, A.; Murrel, K.D.** "Evaluation of Excretory-Secretory Antigens for the serodiagnosis of swine trichinelosis". *Vet. Parasitol.*, 30, 1988, 131-137.
7. **Kim, C.W.; Lebbdetter, M.C.** "Detection of specific antigen-antibody precipitates on the surface of *Trichinella spirallis* by scanning electron microscopy, in *Trichinellosis*" (ed. Kim, E.J., Ruitenberg and J.S. Teppema). *Chertsey. England.* 1981, 65-69.
8. **Mc Laren, D.J.; Ortega-Pierres, G.; Parkhouse, R.M.E.** "*Trichinella spiralis*: inmunocytochemical localization of surface and intracellular antigens using monoclonal antibody probes". *Parasitology*, 94, 1987, 101-114.
9. **Ortega-Pierres, M.G.; Almond, N.W.; Parkhouses, R.M.E.** "Applications of biochemically defined antigens of *Trichinella spiralis* in host immunity, protection and diagnosis". *Wiado. Parazytol.*, 33, 1987, 423-452.
10. **Ortega-Pierres, G. Chayen, A.; Clark, N.W.T.; Parkhouse, R.M.E.** "The occurrence of antibodies to hidden and exposed determinants of surface antigens of *Trichinella spiralis*". *Parasitology*, 88, 1984, 359-369.
11. **Parkhouse, R.M.E.; Philipp, M.; Ogilvi, B.M.** "Surface antigens of *Trichinella spiralis* infective larvae". *Parasite Immunol.*, 3, 1981, 339-352.
12. **Pritchard, D.D.I.** "Antigen production by encysted muscle larvae of *Trichinella spiralis*". *J. of Helminthol.*, 59, 1985, 71-77.
13. **Roth, J.** "The protein A-gold (pAg) technique. A qualitative and cuantitative approach for antigen localization on thin sections", in *Techniques i immunocytochemistry*, (ed. G.R. Bullock and P. Petrusz), Academic Press, London, 1982, Vol. 1, 107-133.
14. **Roth, J.** "The protein A-gold technique for antigen localization in tissue sections by light and electron microscopy", in *Immunolabelling for electron microscopy*, (ed. J.M. Polak and D. Varndell), Elsevier Science Publischers B.V., Amsterdam, 1984, 113-121.
15. **Silberstein, D.S.** "Antigens", in *Trichinella and Trichinosis* (ed. W.C. Campbell), Plenum press, New York, 1983, 309-334.
16. **Silberstein, D.S.; Despommier, D.D.** "Antigens from *Trichinella spiralis* that induce a protective response in the mouse". *J. of Immunol.*, 132, 1983, 898-904.
17. **Sternberger, L.A.** *Immunocytochemistry*, 1979, 2nd Edn. J. Wiley and Sons Inc., New York, 1979.
18. **Takahashi, Y.; Uno, N.; Mizuno, N.; Yamada, S.; Araki, T.** "Ultrastructural localization of antigenic subtances in *Trichinella spiralis*". *Parasitol. Res.*, 75, 1989, 316-324.

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