Immunoparasitological parameters of the intestinal phase of trichinellosis in rats

G. G. NUÑEZ, S. N. COSTANTINO *and* S. M. VENTURIELLO*

Humoral Immunity Studies Institute, CONICET, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Junín 956, (1113) Buenos Aires, Argentina

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SUMMARY

Enzyme-linked immunosorbent assays (ELISAs) were developed in order to detect coproantigens (CAgs), coproantibodies (CAbs) and faecal immune complexes (FIC) in rats experimentally infected with *Trichinella spiralis*. The usefulness of these assays was compared to that of a conventional ELISA for detection of serum antibodies (Abs) to muscle larvae excretory–secretory products (ML–ESP). The ELISA for CAgs was the first parameter to give a positive result but the detection was limited only to day 2 p.i. CAbs against ML–ESP and adult worm excretory–secretory products (AW–ESP) was first positive on day 4 p.i. Anti-ML–ESP remained positive until day 12 p.i. while CAbs against AW–ESP remained positive throughout the study period. Specific IgE and IgA were found. FIC were detected between days 2 and 8 p.i. Serum Abs began to appear on day 10 p.i. Therefore, the ELISA for CAbs was a suitable assay for the detection of the enteral and early phases of the infection.

Key words: coproantigens, coproantibodies, rat trichinellosis, immune complexes.

INTRODUCTION

Diagnosis of *Trichinella spiralis* infection has long been controversial in the sense that the early phase is difficult to detect due, in part, to the non-specificity of the signs and symptoms of infection (Murrell & Bruschi, 1994).

Diagnostic methodologies for this parasite infection have been developed, all of them aimed to detect either the host's immune response, the parasite's products or the parasite itself (Coltorti, 1981; Murrell & Bruschi, 1994; De La Rosa *et al*. 1996). As a rule, none of them has been able to detect the early phase of the infection, the time at which, an anti-parasite drug treatment would be most effective.

It is known that the muscle larvae (ML) of *T. spiralis* release soluble (glyco)proteins, called excretory–secretory products (ESP), into the bloodstream. These larval proteins bear the so-called TSL-1 group of antigens (Ags) which have been demonstrated to be highly immunogenic. Moreover, specific anti-*T. spiralis* antibodies (Abs) can also be produced locally in the gut after infection (Negrão-Corrêa, Adams & Bell, 1996). Therefore, to achieve an early diagnosis of this infection it is important to know the kinetics of parasite Ag release as well as the kinetics of synthesis and specificity of immunoglobulins secreted by the intestinal epithelium after infection.

* Corresponding author: Cátedra de Inmunología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, (1113) Buenos Aires, Argentina. Tel: +54 11 4964 8259/8260. Fax: +54 11 4964 0024. E-mail: sventuri@huemul.ffyb.uba.ar

Taking this into account, the aim of this work was to detect the enteral phase of infection by developing enzyme-linked immunosorbent assays (ELISA) for the detection of coproantigens (CAgs), anti-ML–ESP and anti-adult worm (AW)–ESP coproantibodies (CAbs) and faecal immune complexes (FIC) in rats experimentally infected with *T. spiralis*. The kinetics of these parameters was compared to that of serum Abs against ML–ESP.

MATERIALS AND METHODS

Animals

Five 2-month-old specific pathogen-free Wistar rats were orally infected with 5000 muscle larvae per rat. The muscle larvae were obtained by the digestion method (Larsh & Kent, 1949). Another group of 3 animals remained uninfected as a control group. Animals were kept in individual cages throughout the experiment and given water and food *ad libitum*.

Preparation of faecal extracts

Stool samples were collected during 24 h every 2 days using protease inhibitors (Complete, Boehringer, Mannheim, Germany) starting on day 0 p.i. until day 20 p.i. Individual faecal extracts were prepared following a modified protocol used by Espino & Finlay (1994). Briefly, samples were weighed and thoroughly homogenized with 3 vols of cold phosphate-buffered saline (PBS) plus 0·05% Tween-20 (PBST). Homogenates were then centrifuged for 15 min at 900 ***g*** at 4 °C. Supernatants were collected, aliquoted and kept at −20 °C until used.

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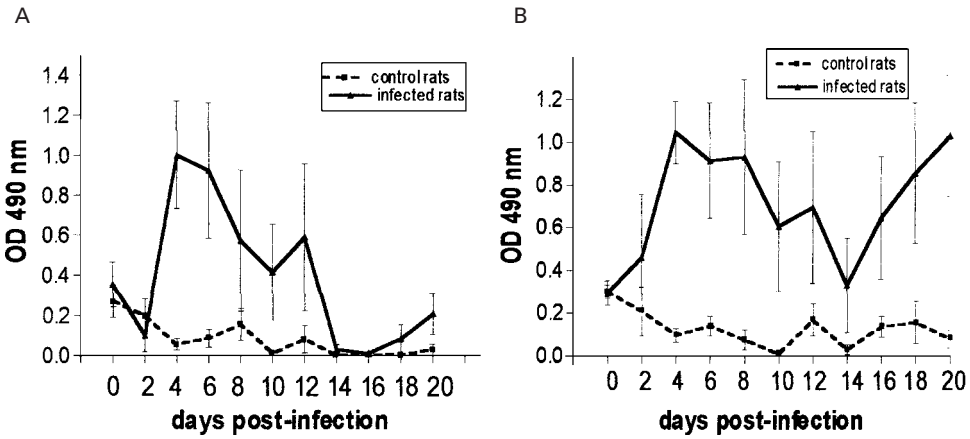

Fig. 1. Detection of anti-ML–ESP (A) and anti-AW–ESP (B) coproantibodies during *Trichinella spiralis* rat infection by ELISA. Each time-point represents mean ± S.E. of 5 animals (infected group) or 3 animals (control group).

Serum samples

In order to detect serum antibodies to ML–ESP, each rat was anaesthetized and bled by retro-orbital puncture on days 0, 5, 10 and 19 p.i. Samples were centrifuged and sera separated, aliquoted and kept at −20 °C until used.

Preparation of adult worm excretory–secretory products

AW excretory–secretory products (AW–ESP) were prepared in our laboratory according to the following protocol. Adult worms were recovered from the intestine of 2-month-old rats orally infected with 7000 muscle larvae 4 days before. Worms were washed 3 times with Eagle's minimum essential medium with Earle's salts (MEM, Gibco, NY, USA) supplemented with antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/ml; Sigma, St Louis, MO, USA), cultured for 18–20 h at 37 °C in an atmosphere containing 5% CO_2 in MEM supplemented with antibiotics (penicillin 50 IU/ml, streptomycin 50 μg/ml; Sigma) at a concentration of 3000 adult worms/ml. After incubation, culture supernatant was collected, filtered through a 0·22 μm membrane (ETC, Darmstadt, Germany) and dialysed against 0·9‰ (w/v) NaCl (saline solution) at 4 °C. Protein concentration was determined by the Bradford procedure (Bradford, 1976) and the ratio of optical densities (OD) at 280 and 260 nm calculated to ensure the absence of any bacterial or fungal contamination. AW–ESP were analysed under reducing conditions using dithiothreitol by SDS–PAGE: 4% stacking gel and 12·5% running gel, according to Laemmli (1970) and gels silver-stained. AW–ESP were aliquoted and kept at −20 °C until used.

ELISA for serum antibodies

Serum Abs were detected by indirect ELISA. Briefly, flat-bottomed PolySorp polyvinyl microtitre plates (Nunc, Roskilde, Denmark) were coated with ML–ESP, obtained as previously described (Gamble *et al.* 1983), at a concentration of 5 μg/ml in carbonate buffer, pH 9·6, during 1 h at 37 °C and overnight at 4 °C. After coating, and between each step, plates were washed 3 times with PBST. Plates were then blocked using 3% non-fat dry milk in PBS (PBSM) for 1 h at 37 °C. Rat serum samples were tested diluted 1/10 in PBST plus 0·3% non-fat dry milk (dilution buffer). After incubating 1 h at 37 °C a horseradish peroxidase-conjugated anti-rat IgG serum (Pasteur, Marnes-La-Coquette, France) suitably diluted was added and incubated for 1 h at 37 °C. Colour reaction was developed using 0·004% (w/v) *o*-phenylendiamine (OPD, Sigma) and 0·004% (v/v) H_2O_2 in citrate buffer, pH 5·0. Reaction was stopped by addition of 4 M H_2SO_4 and the OD read at 490 nm in an ELISA reader (Metertech Σ960). Samples were run in duplicate.

ELISA for coproantibodies

Total CAbs were detected by an indirect ELISA in microtitre plates coated with ML–ESP or AW–ESP as above. Plates were blocked as above and all incubations were carried out at 37 °C with 6 washes with PBST in between. Faecal extracts were tested undiluted. As secondary antibody, a biotinylated anti-rat gammaglobulin serum (Vector, Burlingame, CA, USA) suitably diluted was added, followed by a macromolecular complex of avidin and biotinylated peroxidase (ABC, Vector) according to the manufacturer's directions.

Isotypes of specific CAbs were detected by capture (IgE) and indirect ELISA (IgG, IgM and IgA). For the detection of specific IgE, an anti-rat IgE serum diluted 1/500 (Bethyl, TX, USA) was used as capture antibody. After incubating with each sample, the reaction was continued by the addition of ML–ESP (4 μg/ml) and a human anti-*T. spiralis* gammaglobulin fraction (100 μg/ml). As detection antibody an anti-human gammaglobulin serum

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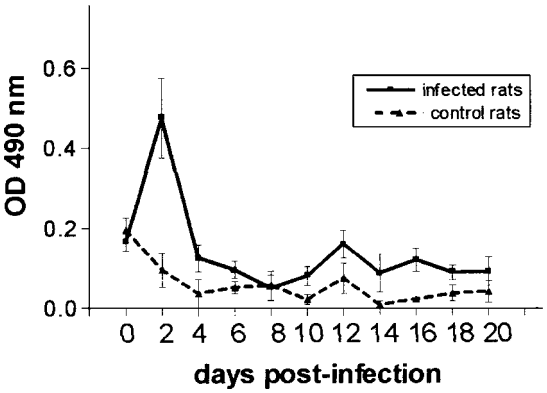

Fig. 2. Detection of coproantigens during *Trichinella spiralis* rat infection by ELISA. Each time-point represents mean ± S.E. of 5 animals (infected group) or 3 animals (control group).

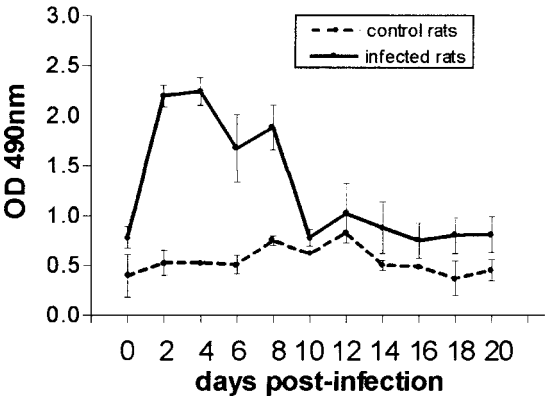

Fig. 3. Detection of faecal immune complexes during *Trichinella spiralis* rat infection by ELISA. Each time-point represents mean ± S.E. of 5 animals (infected group) or 3 animals (control group).

conjugated to peroxidase (Biosys, Compiègne, France) was used. Indirect ELISAs for IgG, IgM and IgA were carried out in the same fashion as total CAbs, employing the following sera: anti-rat γ chain, anti-rat μ chain and anti-rat α chain and the corresponding anti-species peroxidase conjugates (Bethyl). Colour reactions were developed as above. Samples were run in duplicate.

ELISA for coproantigens

CAgs were detected by capture ELISA. Monoclonal Ab PG6B1, with reactivity against the 45–55 kDa components of the ML–ESP, kindly provided by Dr Bruschi was used to coat microtitre plates at a concentration of 5 μg/ml in carbonate buffer, pH 9·6. Plates were blocked as above and all incubations were carried out at 37 °C with 6 washes with PBST in between. Faecal extracts were added undiluted. After the faecal extract incubation, a human anti-*T. spiralis* serum, suitably diluted in PBST, was added and incubated. An anti-human serum conjugated to peroxidase (Biosys) was then added. The colour

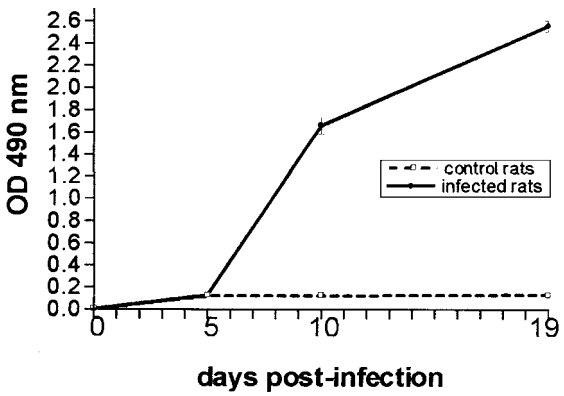

Fig. 4. Detection of serum antibodies to *Trichinella spiralis* ML–ESP by ELISA. Each time-point represents mean ± S.E. of 5 animals (infected group) or 3 animals (control group).

reaction was developed and stopped as above. A standard curve of ML–ESP diluted in normal rat faecal extract ranging from 20 to 10 000 ng/ml was run in each plate. All samples were run in duplicate.

Faecal immune complexes (FIC)

Precipitation. One vol. of faecal extract was mixed with 1 vol. of 6% polyethylene glycol 8000 (PEG, ICN, CA, USA) in borate buffer, pH 8·5. Mixtures were then incubated overnight at 4 °C and then centrifuged 1 h at 900 ***g*** at 4 °C. Precipitates were then washed 3 times with 3% PEG and the final pellet resuspended in PBS to a final volume equivalent to 1/10 of the initial volume. No free Ag was precipitated under these experimental conditions.

Detection. FIC were detected by capture ELISA. A human anti-*T. spiralis* gammaglobulin fraction, was used to coat microtitre plates at a concentration of 160 μg/ml in carbonate buffer, pH 9·6 (1 h at 37 °C and overnight at 4 °C). Plates were blocked using 10% non-fat dry milk in PBST for 1 h at 37 °C. All steps were followed by 3 washes with PBST. Precipitated FIC were added and incubated 1 h at 37 °C and overnight at 4 °C. A biotinylated anti-rat gammaglobulin serum (Vector) plus 20% normal human serum was then added followed by the ABC. Colour reaction was developed and stopped as above. A PEG-precipitated stool extract from a normal rat served as negative control. For the positive control an FIC preparation was made by mixing ML–ESP with an anti-*T. spiralis* rat gammaglobulin fraction in normal faecal extract in a ratio previously determined. All samples were run in duplicate.

RESULTS

Detection of coproantibodies

Fig. 1A and B shows the kinetics of appearance of CAbs to *T. spiralis* ML–ESP and AW–ESP

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respectively. As it can be observed, CAbs anti-ML–ESP appeared as early as day 4 p.i., displaying a maximum level on days 4–6 p.i. and remaining at detectable levels until day 12 p.i. When AW–ESP were used, it was observed that positive reactions could also be found on day 4 p.i. but, in this case, the response was more sustained, with animals remaining positive until at least day 20 p.i. The isotypes of specific immunoglobulins were determined at the peak values of total CAbs. The presence of IgA and IgE to *T. spiralis* could be demonstrated (OD values for IgA: $0{\cdot}457 \pm 0{\cdot}110$ and $0{\cdot}118 \pm 0{\cdot}027$ infected versus control group and OD values for IgE: $0{\cdot}484 \pm 0{\cdot}053$ and $0{\cdot}167 \pm 0{\cdot}009$ infected versus control group), whereas neither IgG nor IgM could be detected.

Detection of coproantigens

In order to determine the presence of parasite antigen in faecal extracts a capture ELISA was used. It was observed that mAb PG6B1 was able to detect considerable levels of antigen in all the animals only on day 2 p.i., being the average level of 234 ± 136 ng/ml (Fig. 2). The analytical sensitivity of the assay was approximately 40 ng/ml.

Detection of faecal immune complexes

FIC were detected at levels distinguishable from non-infected rats between days 2 and 8 p.i. (Fig. 3).

Detection of serum antibodies

Serum samples were tested at low dilutions in order not to miss any positive reaction. However, serum antibodies to *T. spiralis* were detected from day 10 p.i. onwards (Fig. 4). No positive reactions were found in control animals.

DISCUSSION

In this work ELISAs were developed in order to assess immunoparasitological parameters of the enteral phase of trichinellosis in the rat. Both anti-ML–ESP and anti-AW–ESP Abs could be detected in faecal extracts from day 4 p.i. with these Abs belonging to the IgA and IgE classes. These findings are in agreement with previous works that demonstrated an early synthesis of antibodies in the gut (Negrão-Corrêa, Adams & Bell, 1996, 1999). On the other hand, the kinetics of synthesis of anti-AW–ESP antibodies was more sustained over time. This fact might account for the transformation of muscle larvae into adult worms, which can occupy the intestinal mucosa for up to 14 days in the rat, thus being able to stimulate the immune system during a longer period.

Since CAbs can recognize both ML–ESP and AW–ESP antigens it could be speculated that there exist antigenic determinants which are shared between these parasite stages or that at the beginning of the infection there are 2 or more co-existing antibody subpopulations having different specificities. Besides, our results also show that from day 14 p.i. on, the antibodies present in faecal extracts only recognize epitopes present in the AW–ESP, thus showing that the AW–ESP has specific antigens as well. The fact that the ELISA for CAbs rendered positive results earlier than the ELISA for serum antibodies would be of particular importance in the case of human infections to achieve an early diagnosis which, in turn, allows the administration of a more effective antihelminthic treatment.

Despite the presence of live parasites in the intestinal tract (Nuñez *et al*. 2002), the ELISA for CAgs rendered positive results only at the very onset of the infection (day 2). Several reasons can explain this finding. Firstly, it is possible that the amount of antigen released by the parasite is too low to be detected, even when the system can detect concentrations of about 40 ng/ml. This sensitivity could not be improved by the utilization of a capture polyclonal gammaglobulin fraction obtained from a pool of sera belonging to individuals naturally infected (data not shown). Boulos *et al*. (2001) developed a system which offers a longer detection period, but in this case the capture antibody was obtained by immunization of rabbits with crude extracts of the muscle larvae. Secondly, it must be recalled that the capture mAb used in this assay is directed to the TSL-1 antigens present in the ML–ESP and it is known that the AW–ESP stage lacks this group of antigens (Ortega-Pierres *et al*. 1996). Finally, it is probable that negative results are due to immune complex formation. In fact, FIC appeared on day 2 p.i., the only time-point at which CAgs could be detected and when CAbs were still undetectable.

To sum up, this work demonstrates that during *T. spiralis* infection, CAgs, CAbs and FIC can be detected in faecal samples and suggests that the detection of CAbs can be a useful tool for an early detection of this parasite infection.

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