DETECTION OF TYROSINE PHOSPHORYLATED PROTEINS IN *TRICHINELLA SPIRALIS* L1 LARVAE

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Summary :

Western-blotting analysis showed the presence of tyrosine phosphorylated proteins in crude extracts of *T. spiralis* larvae and these phosphorylated proteins were located by immunofluorescence on the striations of the larval cuticle. The patterns of phosphorylated proteins were modified when larvae were incubated with bile.

KEY WORDS : phosphorylated protein, *Trichinella*, tyrosine.

The short intestinal phase of the parasitic nematode *Trichinella* is characterised by a rapid transformation in less than 36 hours of L1 larvae into adults. The biochemical changes in the intestinal environment of the larvae liberated from the nurse cell by pepsin digestion, are followed by a shift from a coiled to a serpentine morphology of the larvae (Stewart *et al.*, 1987) and the by the shedding of the accessory and external layer of the cuticle, as seen when L1 larvae undertake their first moult (Wright *et al.*, 1988). Modha *et al.* (1995), using a technique of caged-compounds, showed the role of second messengers (such as calcium, AMPc, IP3...) in these changes and proposed an hypothetical signal transduction pathway involved in the modification of *T. spiralis* larvae surface during intestinal activation.

As calcium seemed to play a role in these morphological changes, we hypothetized that tyrosine phosphorylated proteins and/or receptors could be implicated in this process. In this study, we searched for tyrosine phosphorylated proteins in crude extracts of *T. spiralis* larvae.

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MATERIALS AND METHODS

PARASITE

The *T. spiralis* (TRLL, ISS 104) strain used in these experiments was isolated during a horse meat related outbreak of 1985 and maintained in mice. The L1 larvae were obtained from OF1 Swiss male mice, infected 14 weeks previously, by digestion in 0.5 % HCl-pepsin for two hours. The larvae were washed several times with distilled water and re-suspended in 0.9 % NaCl.

PREPARATION OF *TRICHINELLA* CRUDE EXTRACTS

Crude extracts were prepared with 3,000 larvae obtained after HCl-pepsin digestion. Extracts were prepared from larvae freshly obtained after muscle digestion or from larvae treated with bile. In this last case, larvae were incubated at 37° C for 5, 15, 60 and 240 minutes in Petri dishes containing 3 ml of RPMI 1640 medium at pH 7.4 (R-61870-010, Gibco BRL, Eragny, France) and 5 % biliary acids 450-100 (Sigma, St-Quentin-Fallavier, France). Larvae were frozen at – 20° C. After thawing, they were washed several times with distilled water, re-suspended in buffer (2 % sodium dodecyl sulfate SDS, 10 % glycerol, 0.15 M Tris, 2 µM vanadate and 6 µM PMSF) and ground on ice with a minihomogenizer. The protein content was determined by the Lowry method. Then, 5 % 2-mercaptoethanol were added and proteins were analysed by electrophoresis through a 12 % polyacrylamide gel (Serva, Saint-Germain-en-Laye, France) and transferred to nitrocellulose membrane (Transphor, Hoefer Scientific Instruments, San Francisco, California), as previously described (Dupouy-Camet *et al.*, 1988). The membranes were blocked with Tris buffered saline (0.05 M Tris, 0.15 M NaCl) containing 1 % Tween and 1.2 % gelatin (Sigma, St-Quentin-Fallavier, France).

ANTIBODIES

Nitrocellulose membranes containing *Trichinella* proteins were first incubated with different monoclonal anti-phosphotyrosine antibodies prepared from mice

and diluted 1:1000 in TBS-Tween: 1G2 and PY20 (Santa Cruz Biotechnology, England), 4G10 (Sigma, St-Quentin-Fallavier, France). Nitrocellulose membranes were then incubated with an anti-mouse immunoglobulin-peroxidase conjugate diluted 1:10000 and phosphorylated proteins were revealed by luminol-autoradiography using the ECL kit (Amersham Life Science, UK).

IMMUNO-LOCALISATION OF TYROSINE PHOSPHORYLATED PROTEINS

Frozen sections of parasitised muscle, embedded in mouse liver were prepared for immunofluorescence studies with the monoclonal 1G2 diluted 1:50 in TBS containing 2 % glycine and skimmed-milk (Régilait, Lyon, France). Antibody binding was assayed by a fluoresceine conjugated anti-mouse IgG (Sigma, St-Quentin-Fallavier, France) diluted 1:200 in TBS containing 2 % glycine and skimmed-milk. After two washes with a phosphate buffer at pH 7.2, sections were examined under UV light (Leitz Laborlux 12).

RESULTS

Western-blotting analysis of *T. spiralis* crude extracts with anti-phosphotyrosine monoclonal antibodies showed the presence of tyrosine phosphorylated proteins with a molecular weight ranging from 14 to 103 kDa (Fig. 1). This detection of phosphorylated proteins was obtained with all types of anti-phosphotyrosine antibodies tested (1G2, PY20 et 4G10). When Western blots of crude extracts were incubated in a solution of free tyrosin

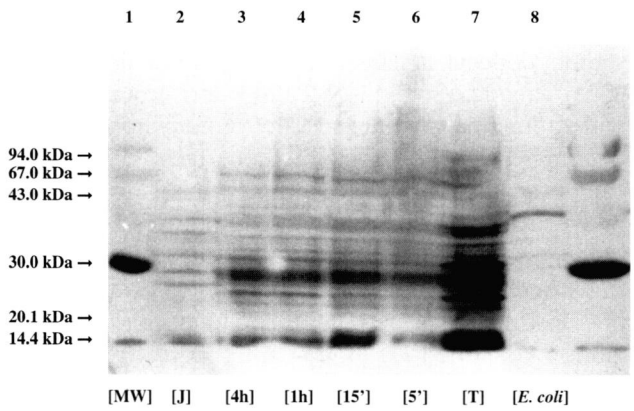

Fig. 1. – Analysis of *Trichinella spiralis* antigens by anti-phosphotyrosine antibody 1G2. with and without bile: lane 1: molecular weight marker; lane 2: Jurkat cells crude extract (postive control); lane 3: extracts of larvae incubated in bile for four hours; lane 4: extracts of larvae incubated in bile for one hour; lane 5: extracts of larvae incubated in bile for 15 minutes; lane 6: extracts of larvae incubated in bile for five minutes; lane 7: *Trichinella* crude extract; lane 8: *E.coli* crude extract (negative control).

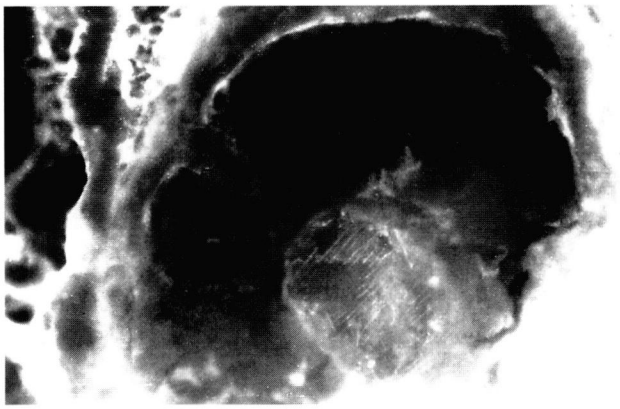

Fig. 2. – Detection by immunofluorescence of tyrosine phosphorylated proteins on the pseudo-segmentation of the cuticle in cryosections of *T. spiralis* larvae in parasitized muscles.

phosphorylated proteins with the anti-phosphotyrosine antibodies, no bands were revealed, thus demonstrating the specificity of antibody binding. Indirect immunofluorescence on frozen sections of mouse diaphragms infected with *T. spiralis* showed that tyrosine-phosphorylated proteins were located on the striations of the larval cuticle (Fig. 2).

Western blot patterns of tyrosine-phosphorylated proteins were modified when larvae were incubated with bile. Bile induced after five minutes of incubation, the decrease of the phosphorylation of a 23 kD protein. This decrease was not observed after 15, 60 or 240 minutes of incubation (Fig. 1).

DISCUSSION

We have shown in these experiments the presence of tyrosine phosphorylated proteins in crude extracts of *T. spiralis* larvae which could be localized by immunofluorescence on the striation of the larval cuticle. Phosphorylated-proteins were described on the surface of *Schistosoma mansoni* (Davies & Pearce, 1995) and could act as receptors transducing signals across the parasite surface membrane (Davies *et al.*, 1998) and also be involved in stages differentiation (Wiest *et al.*, 1991). Arden *et al.* (1997) identified a serine/threonine protein kinase activity in excretory/secretory (ES) products of *Trichinella spiralis* infective larvae by detecting phosphorylation of exogenous and endogenous substrates. There was no evidence for protein tyrosine kinase activity in ES products. In our experiments, the pattern of phosphorylated proteins was modified when larvae were incubated with bile. The biochemical changes in the intestinal environment of the larvae are correlated with a shift from a coiled to serpentine aspect of the larvae (Stewart *et al.*, 1987). These modifications could also

be related to the infectivity for intestinal cells as suggested by ManWarren *et al.* (1997) who showed that the invasion of *in vitro* epithelial monolayers was increased when larvae were incubated in bile or digestive juices before culture. Modha *et al.* (1995) showed that these morphological modifications were abolished by Ca^{2+} chelation but overcome by addition of cAMP. These calcium dependent changes, are compatible with the phospho-inositide pathway which involves phosphorylated proteins and receptors. Our data suggest a role for tyrosine phophorylated proteins in the morphological changes of *T. spiralis* larvae induced by digestive juices and bile.

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