The use of immunocytochemical methods for toxicity assessment in *Tetrahymena pyriformis*

Nicolina DIAS, Ana NICOLAUS, Nelson LIMA, Graça S. CARVALHO, Manuel MOTA

1Centro de Engenharia Biológica, Universidade do Minho, Campus de Gualtar, 4709 Braga Codex, Portugal
2Instituto de Estudos da Criança, Universidade do Minho, 4709 Braga Codex, Portugal
3Instituto de Engenharia Biomédica, Praça Coronel Pacheco nº1, 4050 Porto, Portugal

**Introduction**

In the field of advanced microscopic technologies, fluorescent microscopy has become an important tool in cell biology. Fluorescent dyes are highly sensitive and bind to specific target molecules, without changes in their native conformation. These molecules can be localised and analysed in the cell or even in a whole multicellular organ, without interference from neighbouring structures or substances (GILOH and SEDAT, 1982).

Immunofluorescence is an application of fluorescence microscopy using fluorescently labelled antibodies. The use of antibodies in immunocytoology as markers is an exploitation of the antibody-antigen reaction. Antibodies are immunoglobulin proteins that recognise and bind to specific epitopes on target molecules, the antigen (VRIELING and ANDERSON, 1996). This conjugation is accomplished without destroying any biological or immunological property, and immunofluorescence allows the visualisation of antigen localisation.

Protozoa, like other eukaryotic cells, have a cytoskeleton. Among them, ciliates and flagellates present an additional high level of structural organisation within the cortex, that implies complex patterns in regulation of cytoskeletal proteins. *Tetrahymena pyriformis*, a free-swimming ciliate, presents a robust well-developed cytoskeleton, composed by actin and tubulin; isoforms of tubulin constitute the axonemal proteins, present in basal bodies of cilia and flagella (WILLIAMS et al., 1990; WILLIAMS et al. 1995).

It is widely known that toxic substances become harmful for the living cells before reaching a lethal concentration. Since the molecular components involved in the cytoskeleton often undergo dramatic reorganisation in response to internal and external stimuli, that fluorescent techniques may be expected to be a powerful tool to detect changes in the cytoskeleton proteins of sensitive microorganisms, such as protozoa. In the present paper, immunocytochemical methods were developed to study structural effects of toxic substances in sub-lethal levels in *Tetrahymena pyriformis*. Two monoclonal antibodies were directed against the cytoskeleton: actin and acetylated α-tubulin. DAPI - 4’6-diamino-2-phenylindole hydrochloride was used to stain DNA presented in the nuclei and in the basal bodies.

**Material and Methods**

Organisms and media

All toxicological assays used axenic 18-24 hours cultures of *Tetrahymena pyriformis*. The medium for growth is known as PPY (Proteose Peptone Yeast Extract Medium) in the 1995 Catalogue of Strains of CCAP (Culture Collection of Algae and Protozoa, UK) and contains (in g/L): Proteose Peptone 20.0 and Yeast Extract 2.5; pH was adjusted between 6.6 and 6.8 whenever necessary. All tests were performed at 20±1°C.
Toxics and concentrations

_Tetrahymena pyriformis_ was exposed to different concentrations of copper, zinc, cycloheximide (an antibiotic that inhibits protein synthesis) and Triton X-100 (a neutral surfactant that disrupts cell membrane).

Copper was used in four concentrations: 300 mg/L, 400mg/L, 500mg/L, 600mg/L. Dehydrated copper chloride was used; pH between 6.6 and 6.8 was achieved by addition of 0.1N NaOH.

Zinc was used in four concentrations: 50 mg/L, 100mg/L, 200mg/L and 300 mg/L. Zinc chloride was used; pH between 6.6 and 6.8 was achieved by addition of 0.1N NaOH.

Previously to the toxicity assays, a control assay was performed to investigate the influence of chloride used in copper and zinc assays, in the concentrations indicated above. Sodium chloride was used. No effect was detected.

Triton X – 100 was used in five concentrations: 12.5 mg/L, 25.0 mg/L, 37.0 mg/L, 50.0 mg/L and 75.0 mg/L.

Cycloheximide was used in eight concentrations: 0.0002mg/L, 0.001mg/L, 0.002mg/L, 0.01 mg/L, 0.05 mg/L, 0.1 mg/L, 0.5 mg/L and 1.0 mg/L.

Immuno-fluorescence assays

The method was based on SANDERS and SALISBURY (1995) with some modifications.

Living cells were immobilised in 16-well epoxy-coated microslides for easy handling in subsequent steps. Pre-treatment of wells was made with Polyethilenimine 0.1% to promote adhesion of cells. After 30 seconds, the wells were rinsed with deionized water and were allowed to air-dry. Polyethilenimine-treated slides were used within one hour. Cells were allowed to settle onto the bottom of the wells for 30 minutes and were fixed with absolute methanol pre-cooled at -20°C. After 5 minutes, they were washed, to rehydrate, three times, 5 minutes each, with Phosphate Buffer Saline (PBS). Triton X-100 0.1% was used in the first wash to help to permeabilise the cell membrane and promote extraction of components that otherwise would contribute to unwanted levels of background labelling.

Indirect immuno-fluorescence assay was started by placing one drop of the primary antibody in each well for 90 minutes. This primary reagent was a monoclonal antibody, either anti-actin (Sigma A-1804) diluted 1:400 in PBS solution or anti-acetylated α-tubulin (Sigma T-6793) diluted 1:200 in PBS solution. Following the incubation, slides were washed three times, 5 minutes each, in PBS solution again. Secondary antibody, fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG (Sigma F-5262), diluted 1:400 in PBS, was added, staining the cells. Slides were incubated for 1 hour. After staining, preparations were washed as before. Slides were then mounted in mounting medium (Sigma1000-4). Immediately after, one drop of 0.1 μg/mL DAPI was added to stain DNA. Coverslips were sealed with nail polish.

Observations were performed using a Zeiss Axioskop microscope equipped with epifluorescence: filter set EX450/490, FT510, LP520 for FITC visualisation, filter set EX365, FT395, LP420 for DAPI and filter set EX395/440, FT460, LP470 for simultaneous FITC and DAPI visualisation. Photographs were taken with a microscope camera Zeiss MC 100, using Fujichrome SENSIA film, ASA 400.

Results and Discussion

Actin was found all over the cell but not in the nutrition vacuoles nor in the nucleus. There was no evidence for the presence of actin in cilia (FIGURE 1).
Acetylated α-tubulin was found in basal bodies and cilia. Fluorescence in oral apparatus was more intense, suggesting an higher number of basal bodies and cilia in this region. In normal cells, basal bodies were arranged in rows along the body (FIGURE 2), whereas after exposition to toxics, such as cycloheximide, they changed their spatial organisation (FIGURE 3). Acetylation is the post-translational modification of α-tubulin that allows stabilisation of the microtubules. Acetylated α-tubulin occurs in cilia and cytoskeleton, when microtubules are in their assembled form, but it is not found in cytoplasmic pools of disassembled soluble tubulin (SULLIVAN, 1988; PENQUE et al., 1991).

FIGURE 1- Actin in T. pyriformis cells marked by anti-actin monoclonal mouse antibody followed by FITC anti-mouse IgG (ampl. 800X; 450/490 nm).

FIGURE 2-Alpha-tubulin in T. pyriformis cells marked by anti-acetylated α-tubulin monoclonal mouse antibody followed by FITC anti-mouse IgG showing basal bodies arrangement in normal cells. (ampl. 800X; 450/490 nm).

FIGURE 3-Alpha-tubulin in T. pyriformis cells marked by anti-acetylated α-tubulin monoclonal mouse antibody followed by FITC anti-mouse IgG showing cytoskeleton disarrangement in cells exposed to cycloheximide (ampl. 1200X; 450/490).
DAPI stained very strongly basal bodies, but did not stain cilia (FIGURE 4). Since DAPI is a specific dye for DNA, these results suggest that each basal body is associated to DNA. This observation is sustained by related studies (HALL et al., 1989; RANDALL and DISBREY, 1965).

![Fluorescence micrograph of T. pyriformis cells stained with DAPI showing basal bodies arrangement in normal cells. (ampl. 800X; 395/440 nm)](image)

**FIGURE 4.** Fluorescence micrograph of *T. pyriformis* cells stained with DAPI showing basal bodies arrangement in normal cells. (ampl. 800X; 395/440 nm)

*Tetrahymena pyriformis* is a very simple eukaryote that is being used as a model to studies in genetics and to elucidate the mechanisms responsible for the functional diversity in microtubules. Recently, ciliates have been used more and more in toxicological tests and this study sustains their potential in standard bioassays.

**Acknowledgements**

Nicolina Dias and Ana Nicolau were supported by grants BM/4291 and BD/5080/95 from PRAXIS XXI, respectively. This project was supported by a PRAXIS XXI-2/2.1/ BIO/1118/95 contract.

**References**


