Immunocytochemical detection of DNA and RNA in endosymbiont-bearing trypanosomatids

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Abstract

Research about the kinetoplast of trypanosomatids has yielded valuable information about the organization of extranuclear structure. However, the ultrastructural localization of nucleic acids within these protozoa remains uncertain. We have applied cytochemical and immunocytochemical approaches to precisely identify DNA and RNA in lower endosymbiont-bearing trypanosomatids. Using the Terminal deoxynucleotidyl Transferase (TdT) immunogold technique, we showed that nuclear DNA is seen associated with the nuclear envelope during the trypanosomatid cell cycle. By combining the TdT technique with the acetylation method, which improves the contrast between structures containing fibrils and granules, we have demonstrated that the nucleolus of endosymbiont-bearing trypanosomatids is composed of two constituents: a granular component and a DNA-positive fibrillar zone. Moreover, we revealed that DNA of endosymbiotic bacteria consisted of electron-dense filaments which are usually in close contact with the prokaryote envelope. Using a Lowicryl post-embedding immunogold labeling procedure with anti-RNA antibodies, we showed the presence of RNA not only over the cytoplasm, the interchromatin spaces and the nucleolus, but also over the kinetoplast and virus-like particles present in Crithidia desouzai. © 2003 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Protozoa of the Trypanosomatidae family include species that cause severe parasitic diseases in humans, animals and plants. These protozoa share the kinetoplast, a unique and characteristic structure that contains up to 30% of the total cell DNA organized in a network of interlocked minicircles and maxicircles (reviewed in [1]). Although the description of RNA editing and trans-splicing in the kinetoplast has stimulated studies of extranuclear DNA organization [2,3], the precise nuclear DNA localization during the life cycle of trypanosomatid cells is still a matter of debate. Contrary to all other eukaryotic cells studied so far [4], the nucleolus of trypanosomatids has been described as a homogeneous structure without distinct components [5]. Another limitation in ultrastructural analysis of the trypanosomatid nucleolus has been the lack of selective RNA detection methods. RNA was previously detected in kinetoplast-enriched fractions isolated from some trypanosomatids [6–8]; however, its presence in situ has not been previously reported.

Furthermore, a few members of this family harbor in their cytoplasm an endosymbiotic bacterium. Ultrathin sections of endosymbiont-bearing trypanosomatids analyzed by transmission electron microscopy have revealed that the symbiont presents a matrix with an electron-dense area, mainly composed of ribosomes, and an electron-lu-cid zone, formed by filaments (reviewed in [9]). Finally, one of these lower trypanosomatids, Crithidia desozai, also possesses virus-like particles (VLPs) [10], but the nature of the genetic material present in these particles is still unknown. In order to shed light on the presence of nucleic acids within different structures of lower trypanosomatids, we have applied cytochemical and immunocytochemical
approaches for precisely identifying DNA and RNA at the ultrastructural level. For DNA detection, we have used the in situ Terminal deoxynucleotidyl Transferase (TdT) immunogold technique [11]. This method relies on the fact that DNA ends generated during sectioning are elongated using exogenous TdT and triphosphate bromodeoxyuridine. Subsequently, the labeled sites present at the surface of ultrathin sections are visualized by an indirect and very sensitive immunogold labeling procedure with an anti-bromodeoxyuridine antibody and a second colloidal-gold-coupled antibody. The TdT method is compatible with various fixation and embedding conditions and therefore offers the possibility of studying, with high resolution, the precise location of DNA in very well-preserved structures. In order to facilitate the identification of cellular sites containing DNA, the TdT method has been applied to acetylated material [12]. Although the reaction mechanism of this cytochemical technique is unknown, it provides a very clear distinction of different nucleolar components and of ribosomes and gives a high contrast of the condensed chromatin [13]. For RNA detection, we have used a Lowicryl post-embedding immunogold labeling technique, involving anti-RNA antibodies and secondary antibodies coupled to colloidal-gold particles [14].

The results revealed that the nuclear DNA was seen associated to the nuclear envelope during the trypanosomatid cell cycle. The cytochemical and immunocytochemical approaches showed that the nucleolus is composed of fibrillar and granular constituents which are seen distributed in distinct zones. The presence of DNA was also demonstrated in the fibrillar component of the nucleolus, in the fibrous part of the endosymbiotic bacterium and in the kinetoplast. Finally, our results indicate that RNA is present in the kinetoplast and in VLPs of *Crichidia desouzai*.

## 2. Materials and methods

### 2.1. Micro-organisms

*Blastocrithidia culicis*, *Crichidia deanei* and *C. desouzai* were grown in Warren’s culture medium [15] supplemented with 10% fetal calf serum for 24 h at 28°C.

### 2.2. Electron microscopy

*C. deanei* and *B. culicis* were fixed for 60 min at 4°C in 1.6% glutaraldehyde diluted in Sörensen’s buffer (pH 7.4) and acetylated as previously described [13]. After fixation, cells were dehydrated in ethanol and soaked overnight at room temperature (RT) in 95% ethanol:Epon (1:1). Cells were embedded in Epon and polymerized for 3 days at 45°C.

*C. desouzai* was fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, dehydrated in ethanol and embedded in Epon, without post-fixation in osmium tetroxide. Alternatively, protozoa were fixed in 0.1% glutaraldehyde, 4% paraformaldehyde and 0.2% picric acid diluted in 0.1 M cacodylate buffer (pH 7.2). Free aldehydes were quenched with 50 mM ammonium chloride and then cells were post-fixed for 2 h at 4°C with uranyl acetate in 15% acetone. Dehydration was performed in 30–90% methanol and then cells were embedded in Lowicryl K4M resin at −20°C. Ultrathin sections were collected on nickel grids and, after the immunocytochemical procedures, were stained with 5% (w/v) aqueous uranyl acetate and lead citrate.

### 2.3. Immunocytochemistry

The TdT immunogold method was used for the localization of DNA. Grids containing thin sections were incubated for 10 min at 37°C at the surface of the following medium: 20 μM 5-bromo-2-deoxyuridine (BudR) triphosphate (Sigma, St. Louis, MO, USA), 100 mM sodium cacodylate (pH 7.2), 2 mM MnCl₂, 10 mM β-mercaptoethanol, 50 μM ml⁻¹ bovine serum albumin (BSA), 125 U ml⁻¹ calf thymus TdT (Boehringer Mannheim, Mannheim, Germany) [11]. Sections were incubated for 10 min at 37°C in the same medium, but supplemented with 4 μM each of dCTP, dGTP and dATP (Gibco BRL, Merelbeke, Belgium). Then the sections were rinsed twice in double-distilled water and incubated for 30 min in phospho-buffered saline (PBS) (0.14 M NaCl, 6 mM Na₂HPO₄, pH 7.2) containing normal goat serum (NGS) diluted 1:30 and 1% BSA, then rinsed with PBS containing 1% BSA. Subsequently, the sections were incubated for 4 h at RT with monoclonal anti-BudR antibody (Becton Dickinson, Mountain View, CA, USA) diluted 1:50 in PBS containing 1% BSA and NGS diluted 1:50. After washing with PBS containing 1% BSA, the sections were incubated at RT for 1 h with goat anti-mouse IgG coupled to colloidal gold (diameter = 5–10 nm; Jansen Life Science, Olen, Belgium), diluted 1:40 in PBS containing 0.2% BSA, pH 8.2. After washing with PBS containing 1% BSA, the sections were rinsed with deionized water.

Some controls were done in order to eliminate any possibility of non-specific labeling. In the first control, TdT or labeled nucleotides were omitted from the TdT incubation medium. In the second control, BudR triphosphate was substituted by BudR monophosphate. In the third control, sections were preincubated at 37°C for 2 h with 1 mg ml⁻¹ DNase (Sigma; Type DN-Ep) in PBS (pH 6.8) containing 7 mM MgCl₂. In the fourth control, sections were preincubated at 37°C for 2 h with 1 mg ml⁻¹ of pyrimidine-specific RNase (Boehringer Mannheim) in 10 mM Tris–HCl (pH 7.4) containing 15 mM NaCl. In the fifth control, the primary antibody was omitted. Finally, sections were incubated with antibody-free gold particles. For the immunocytological localization of RNA two mouse monoclonal anti-RNA antibodies (D444, BWR5)
were applied in this study as previously described [14]. Ultrathin sections were incubated for 25 min in PSBS (34 mM NaCl, 0.7 mM KCl, 20 mM Na2HPO4, 0.4 mM KH2PO4, 1% BSA, pH 7.2) containing NGS and normal rabbit serum (NRS), both diluted 1:30. Then the sections were incubated for 3 h at RT in the presence of RNA-specific antibodies diluted 1:10 in PSBS containing NGS and NRS, both diluted 1:50. After five rinses in PSBS, the sections were incubated for 30 min with goat anti-mouse IgG3 (heavy-chain-specific; Sigma) diluted 1:100 in PSBS containing NGS and NRS, both diluted 1:50. Subsequently, the sections were rinsed four times in PSBS (pH 7.2) and then incubated for 1 h at RT in a medium containing rabbit-goat IgG coupled to 5 nm colloidal-gold particles (Jansen Life Science) diluted 1:50 in PSBS (pH 8.2). Sections were rinsed with PSBS and then with distilled water. The described labeling was applied to both faces of the grids. Control experiments were carried out as previously described [14]. First, the primary and/or secondary antibodies were omitted. Then, the grids were incubated with antibody-free particles and finally the sections were preincubated at 56°C for 2 h with 1 mg ml⁻¹ RNase A (Boehringer).

After all these immunocytochemical procedures, the sections were stained with uranyl acetate and lead citrate to provide sufficient contrast in the observation of the cellular ultrastructure.

3. Results and discussion

3.1. The nucleus

When the TdT method was applied to non-synchronized trypanosomatids, we observed different nuclear DNA distributions. During interphase, the nuclear DNA appeared as condensed chromatin associated with the nuclear envelope (Fig. 1a) and sometimes as a perinucleolar chromatin. In dividing cells distinguished by the presence of an enlarged kinetoplast, the nuclear membrane remained intact and the chromatin is seen dispersed, giving to the nucleus a more homogeneous appearance (Fig. 1b), as described previously for a closed mitosis [16–19]. These observations demonstrate that the trypanosomatid DNA is associated with the nuclear envelope, which never disassembles, since it participates in the division of the genetic material.

In ultrathin sections prepared for electron microscopy using standard procedures, the nucleolus of trypanosomatid cells showed a homogeneous structure (Fig. 1a), as previously described [5,16]. Sometimes, an electron-lucid area was found in the nucleolus. This corresponds to the fibrillar center and contains DNA, being labeled by the TdT technique (Fig. 1a, arrow). Moreover, the nucleolus was generally not encircled by a shell of condensed chromatin and occasionally came into contact with the hetero-

![Fig. 1. Immunodetection of DNA in different phases of the B. culicis cell cycle. a: In the interphasic nucleus, DNA is identified in condensed masses of chromatin (C) as well as the nucleolus (nu). Note that the nucleolus presented an electron-lucid area which was exclusively labeled (arrows). The non-replicative kinetoplast (K) was also labeled by the TdT technique. b: In a dividing protozoon, the labeling obtained by the TdT technique showed that the chromatin (C) was dispersed but remained associated with the nuclear envelope (arrows). Gold particles were also seen over the replicative kinetoplast (K). Scale bars = 0.25 μm.](image-url)
chromatin associated with the nuclear envelope. We have attempted to visualize different nucleolar constituents using the acetylation method, which improves the contrast between the various nucleolar components. This cytochemical technique was combined with the TdT method for detecting DNA. Under these experimental conditions, an intense labeling was observed over the condensed blocks of chromatin (C). In the nucleolus (nu), a precise labeling is observed over the electron-lucid central region (arrow) – B. culicis. The inset to the right of panel a shows the nucleolar periphery of a trypanosomatid subjected to the acetylation method followed by the TdT technique. This region is composed of an inner zone of fibrils (fz) and an outer zone of granules (gz). Labeling was only seen over the perinucleolar condensed chromatin (C) – B. culicis.

b: Using anti-RNA antibodies, labeling was seen in association with the interchromatin space (is) and the nucleolus (nu), especially in its peripheral region – C. desouzai. c: During the mitosis, trypanosomatids displayed the nucleolar constituents (nc) dispersed through the nucleus. When the TdT technique was applied after the acetylation procedure, labeling was only seen over the condensed chromatin associated to the nuclear envelope (C) – B. culicis. Scale bars = 0.25 μm.

These results clearly demonstrated that the nucleolus of trypanosomatids, like that of other eukaryotic cells [12], is formed at least by two distinct regions: a centrally located fibrillar zone surrounded by a granular zone. This observation is consistent with a previous cytochemical study [20] showing that the nucleolar central region in epimastigote forms of T. cruzi was preferentially stained with the silver impregnation method, which is known to specifically label the fibrillar components of the nucleolus. The fibrillar zone sometimes includes a chromatin-containing vacu-
ole. In agreement with previous studies \[4,12\], we have further observed that the fibrillar zone contains DNA. In T. brucei, Selzer et al. \[5\] also mentioned that a few gold particles were detected in the nucleolus using anti-DNA antibodies. By means of anti-RNA antibodies, we also showed that the granular zone is more intensely labeled than the fibrillar one. The nucleolar peripheral region in T. cruzi has been shown to be preferentially labeled when we applied the EDTA method, which identifies RNP-containing structures \[20\]. These results are consistent with the notion that processing of primary rRNA transcripts and ribosome biogenesis are vectorial processes which usually begin in the fibrillar region and continue into the boundary of the nucleolus \[21\].

3.2. The kinetoplast

When the TdT technique was applied to endosymbiontharboring trypanosomatid species, an intense labeling was always seen associated with the kDNA fibrils (Fig. 3a, arrows), thus confirming previous observations carried out in T. brucei \[5\]. When sections were incubated with anti-RNA antibodies, gold particles were found over the kDNA network, suggesting that some RNA molecules may be juxtaposed to the DNA fibers (Fig. 3b, arrows). Sometimes, a weak labeling was observed in the peripheral regions of the kinetoplast. Our results demonstrate for the first time the presence of RNA molecules in the kinetoplast of trypanosomatids. When applied to epimastigote forms of T. cruzi, the EDTA technique has previously detected ribonucleoprotein granules at the kinetoplast lateral extremities; these structures presumably represent ribosome-like structures \[20\].

3.3. The endosymbiont and VLPs

The application of the TdT method after performing the acetylation reaction allowed the unambiguous identification of endosymbionts by visualizing the ribosome size. The ribosomes present in the matrix of the endosymbiotic bacterium were smaller than those found in the cytoplasm of the host trypanosomatid. In addition, a fibrous, densely contrasted material, appeared in the central part of the
symbiotic bacteria (Fig. 4, arrow), sometimes seen as structures that attach to its envelope. The application of the TdT method to acetylated sections allowed the identification of the genetic material in the symbiotic bacteria, the labeling being restricted to the fibrillar electron-dense material (Fig. 4, arrow). This is the first description of the precise localization of the genetic material associated with the endosymbiont of trypanosomatids. Gold particles associated with sites recognized by anti-RNA antibodies were found dispersed over the bacterial matrix (data not shown). Our observations are consistent with previous studies, suggesting that the matrix of the endosymbiont consists of an electron-dense area, where ribosomes are found, and an electron-lucid region constituted by filaments, which thus represent the genetic material [22].

It has been reported that a great variety of parasitic protozoa may present viruses sharing some common features: they are spherical or icosahedral particles, with a mean diameter of 35 nm, and in most cases their genetic material is composed of RNA [23]. Our data show that the VLPs found in the cytoplasm of *C. desouzai* were not labeled by the TdT technique (Fig. 5a). In contrast, we consistently observed gold particles when the sections were incubated in the presence of anti-RNA antibodies (Fig. 5b), thus confirming the ribonucleoprotein content of the VLPs in *C. desouzai* [10].

In conclusion, the immunocytochemical techniques used in the present study allowed for high-resolution detection of specific nucleic acids present in the nucleus, in the kinetoplast, in the endosymbiont and in the VLPs found in lower trypanosomatids. The fact that such trypanosomatids present several nucleic-acid-containing structures make them excellent models to test the specificity of DNA and RNA immunocytochemical techniques.

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