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Immunocytochemical Study of Recombinant Terminal Deoxynucleotidyl Transferase (TdT) Synthesized by Baculovirus-Infected Insect Cells

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ABSTRACT. **The ability to overproduce terminal transferase through recombinant DNA technology should provide alternate means for generating sufficient quantities for structural and mechanistic study of this creative DNA polymerase. In this work we have investigated, at electron mocroscope level, the morphological modification and ultrastructural localization of synthesized human terminal transferase occurring in Sf-9 cells during recombinant baculovirus infection time. The results obtained showed that TdT is localized and stored only at the cytoplasmic level; the nucleus did not show any specific site able to link the neosynthesized TdT. The amount of the enzyme, estimate by immunostaining analysis, increased with the viral infection time. Morphological changes occurring during viral infection consist mainly of variations of cellular surface, different size and shape of cytoplasmic organelles and modification of nuclear components.**

Terminal deoxynucleotidyl transferase (TdT) is a DNA is polymerase able to generate a somatic diversification of the immunosystem (1, 3). For biochemical properties, particularly localization and modulation restricted to the immunosystem, and for its ability to maintain unchanged the aminoacidic sequence during the phylogenesis, TdT constitutes a suitable model for molecular biology studies (3). Since its first identification, many different sources and methods have been used to obtain purified active enzyme. Initialy terminal transferase was purified from the calf thymus gland, producing a proteolytic form containing two different peptides (4). Purification of the enzyme from human TdT-positive cultured leukemic cell lines offers the possibility of obtaining large amounts of the human form of this enzyme. Recently, overproduction of human TdT protein was obtained by cloning and coding the sequence of human TdT into a Baculovirus, where the expression of terminal transferase is under the control of the polyhedrin protein promoter (5, 13). This system, successfully used to overproduce several eukaryotic proteins (9–12, 14–16), makes it possible to obtain human terminal transferase expressing both immunological and enzymatic activity (5). We have previously demonstrated that terminal transferase is bound to the nuclear matrix (7), showing a localization which is , in pre-T and pre-B lymphocytes, related to the cell cycle, and that the modulation of the enzyme is presumably related to the molecular differentiation of positive cells (6, 8 Di Primio and Bollum, data in press). In this paper we describe, through electron microscope immunocytochemistry, the changes in the presence and localization, which occur during the terminal transferase synthesis in Sf-9 cells infected by recombinant Baculovirus, and speculate that the results obtained constitute a basis to better identify the linkage sites and the subcellular distribution of the terminal transferase.

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MATERIALS AND METHODS

Insect cells of Spodoptera frugiperda (Sf-9), cloned by Gale E. Smith and Carol L. Cherry, were obtained from the American Type Culture Collection (Rockville, MD) and grown in TNM-FH medium supplemented with 10% fetal bovine serum and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin) at 27°C. At 24 and 48 hours after infection, cell samples were removed for morphological analysis, carried out by transmission and scanning electron microscopy, and for light and electron microscope immunocytochemistry. Recombinant Baculovirus, Autographa californica Nuclear Polyhedrosis Virus (AcNPV) expressing enzymatically active human TdT was prepared by L.M.S. Chang as described elsewhere (5). The chimeric polyhedrin/human terminal transferase protein produced in the infected insect cells is identical in molecular weight to and shows the same immunological and enzymatic activity as that of in human lymphoblastoid cells (5).

Immunocytochemical studies.

1) Light microscopy. Immunocytochemical analysis at the light microscopy level was carried out as the control for the following electron microscope procedure. The method used is the same as previously described (6) and no difference is observed between the results obtained by light or electron microscope observations. Controls for the specificity of the immunoreactivity at light and electron microscopy levels were carried out omitting either the primary or secondary antibody.

2) Electron microscopy. Cells were fixed in suspension at different times from infection for 3 hours at room temperature with 2% paraformaldehyde in 0.1 M Na-cacodylate buffer, pH 7.6 and then washed several times in the same buffer. The fixed cells were permeabilized for 30 minutes in freshly prepared 0.4% saponin, 2% normal goat serum in cacodylate buffer and then washed using the same buffer containing 2% sucrose and 0.1 M glycine for 3 hours with several changes of the washing buffer. The cells were then incubated overnight at 4°C with 5 μg/ml of rabbit polyclonal anti-terminal transferase in the same buffer. This antibody is an affinity-purified rabbit antibody to homogeneous calf thymus TdT which reacts with both the native protein and denatured peptides (2). The peroxidase conjugate of goat anti-rabbit IgG was applied at 0.5 μg/ml for 4 hours at room temperature. After washing with PBS the cells were incubated for 15 minutes with the DAB: H_2O_2 substrate solution, rinsed in cacodylate buffer and postfixed with 1% OsO_4 in the same buffer for 1 hour at 4°C. The cells were coated with agar and dehydrated with alcohol and toluene before embedding in EPON 812 for 48 hours at 60°C. Thin sections obtained with an LKB microtome were placed on copper grids and stained briefly with lead citrate. Observations were made in a ZEISS 109 TEM at 60 Kv.

Morphological study.

1) Transmission electron microscopy. Cells suspensions were fixed with 1.25% glutaraldehyde in cacodylate buffer, pH 7.6 for 1 hour at 4°C. After rinsing in the same buffer the fixed cells were postfixed with 1% OsO_4 in cacodylate buffer for 1 hour in a dark cold room. The cells were then washed with buffer and double-distilled water and stained overnight using a saturated uranyle acetate aqueous solution. The cell suspensions were then washed, dehydrated with alchol and toluene, coated with agar and then embedded in EPON 812. Thin sections were obtained and observed as described above.

2) Scanning electron microscopy. Sf-9 infected and noninfected cells (negative control) were fixed with 2.5% glutaraldehyde in cacodylate buffer for 2 hours in an ice bath. Fixed cells were then washed in cacodylate buffer and dehydrated in a series of ethanol, dried by the critical point drying method

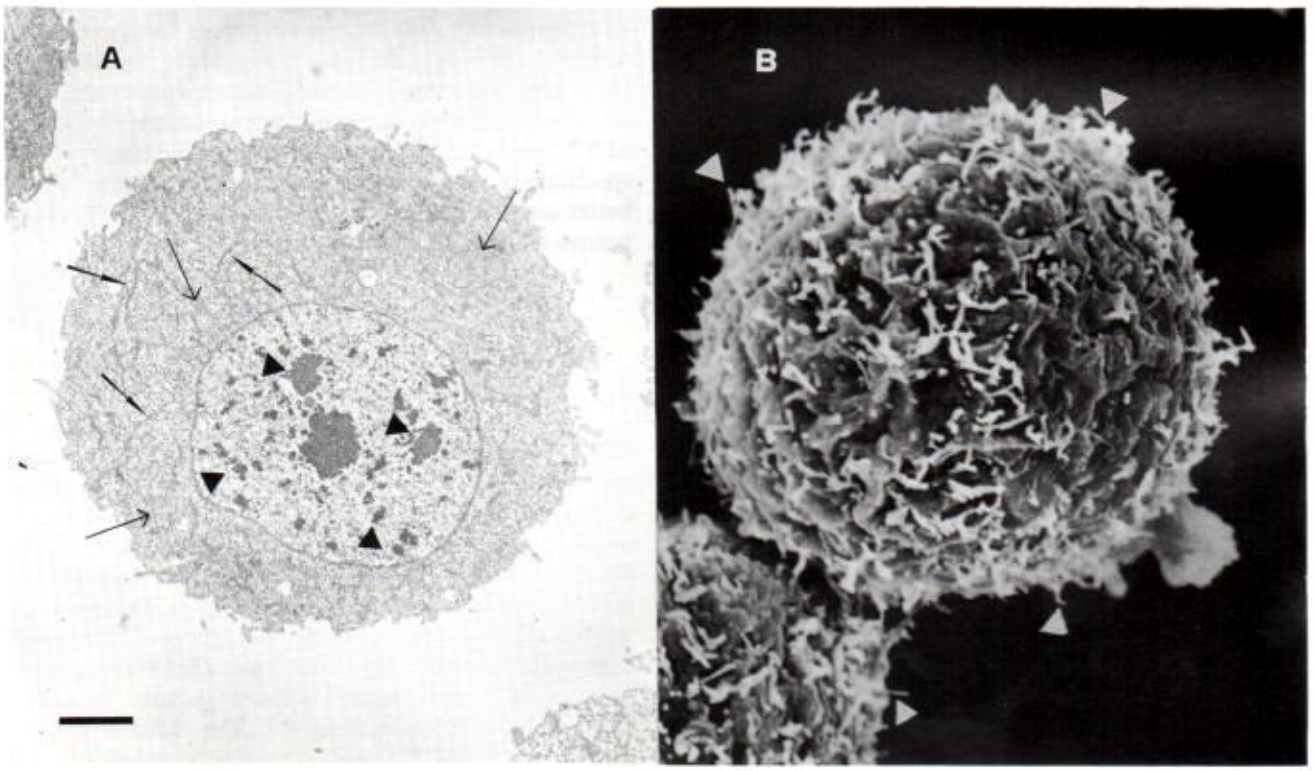

Fig. 1. a) Transmission electron microscope analysis of Sf-9 cells observed before viral infection. Glutaraldehyde/osmium tetroxide-fixed cells. Ultrathin sections obtained from Epon-812-embedded preparation. Thin arrow: Mitochondria; empty arrow: RBR. Scale bar 0.3 μm. b) Scanning electron microscope analysis of same cells as observed in figure a. Glutaraldehyde-fixed gold-coated cells. Arrowheads: microvilli. Scale bar 0.3 μm.

(Balzer BU 101) using liquid carbon dioxide, then mounted on stubs and then sputter-coated with gold in an ion coater. Specimens were examined in the Cambridge S 200 Scanning Electron Microscope.

Source of Materials.

Spodoptera frugiperda (Sf-9) cells infected by the Baculovirus Autographa california Nuclear Polyhedrosis Virus (AcNPV) recombinant system were produced as described (5). Rabbit polyclonal antibody against 32-KDa calf thymus TdT protein was produced as described (2). Peroxidase-conjugated goat anti-rabbit IgG and normal goat serum were from Litton Bionetics (Kensington, Md., USA). Epon 812, glutaraldehyde, lead citrate, osmium tetroxide, and all other reagents for electron microscopy were from Polyscience (Warrington, Pa., USA). Paraformaldehyde, Tris, DAB, Na-cacodylate and all reagent-grade materials were from Sigma (St. Louis, Mo., USA).

RESULTS

In transmission electron microscopy Sf-9 cells reveal (fig. 1a) an irregular surface with membrane blebs and microvilli. The cytoplasm contains few roundish mitochondria, many RER profiles, some lysosomes and vesicles. The nucleus, consisting mainly of euchromatin, shows many small blocks of heterochromatin either dispersed or often bound to the nuclear membrane and one or more nucleoli. Scanning electron microscopy confirms (fig. 1b) the presence of many extroflections and microvilli on the cell surface. Immunocytochemical analysis shows that the cells do not contain TdT. Twenty-four hours after viral infection by Autographa californica Polyhedrosis Virus, several morphological modifications occur (fig. 2a, b). As compared to control cells the cell surface presents larger and flatter extroflections, while the microvilli are reduced (fig. 2b). TEM analysis (fig. 2a) shows that the cytoplasm contains some profiles of smooth and rough endoplasmic reticulum, and many mitochondria with variable shape and size often all situated to one side of the cytoplasm. The immunocytochemical analysis (fig. 4a) shows a fine specific immunoprecipitate localized only at the cytoplasmic level, which is already present in 24-hour-infected cells, indicating that synthesis of TdT began soon after infection. Forty-eight hours after viral infection, the ultrastructural modifications observed at 24 hours appear increased. In fact, the cytoplasm contains mitochondria of different sizes and morphology, many vesicles, lysosomes and viral particles (fig. 3a). At the nuclear level, changes of the chromatin organization and of the nu-

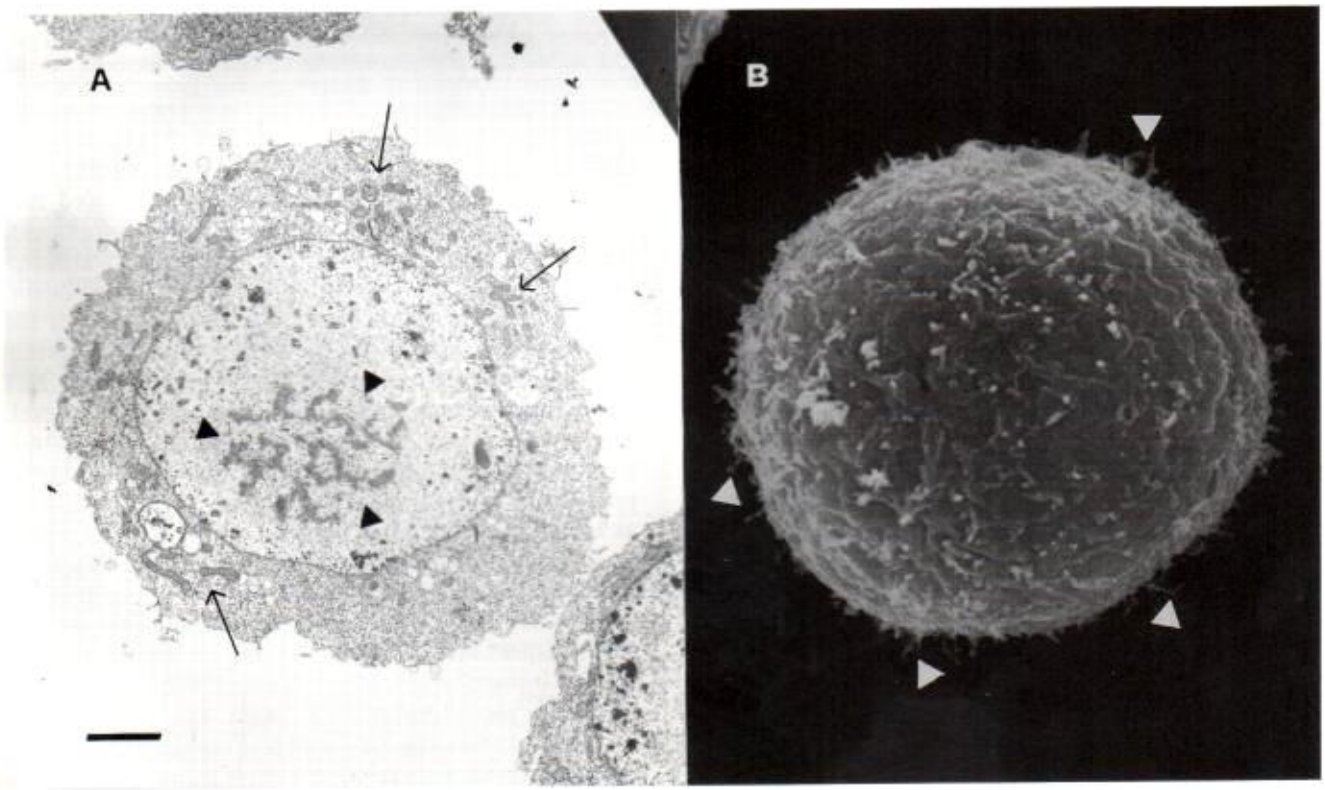

Fig. 2. a) TEM analysis of Sf-9 cells 24 hrs after recombinant baculovirus infection. Thin arrow: mitochondria, arrowhead: NOR. Scale bar 0.3 μm. b) SEM analysis of same preparation of 24-hr-infected cells. Arrowheads: microvilli. Scale bar 0.3 μm.

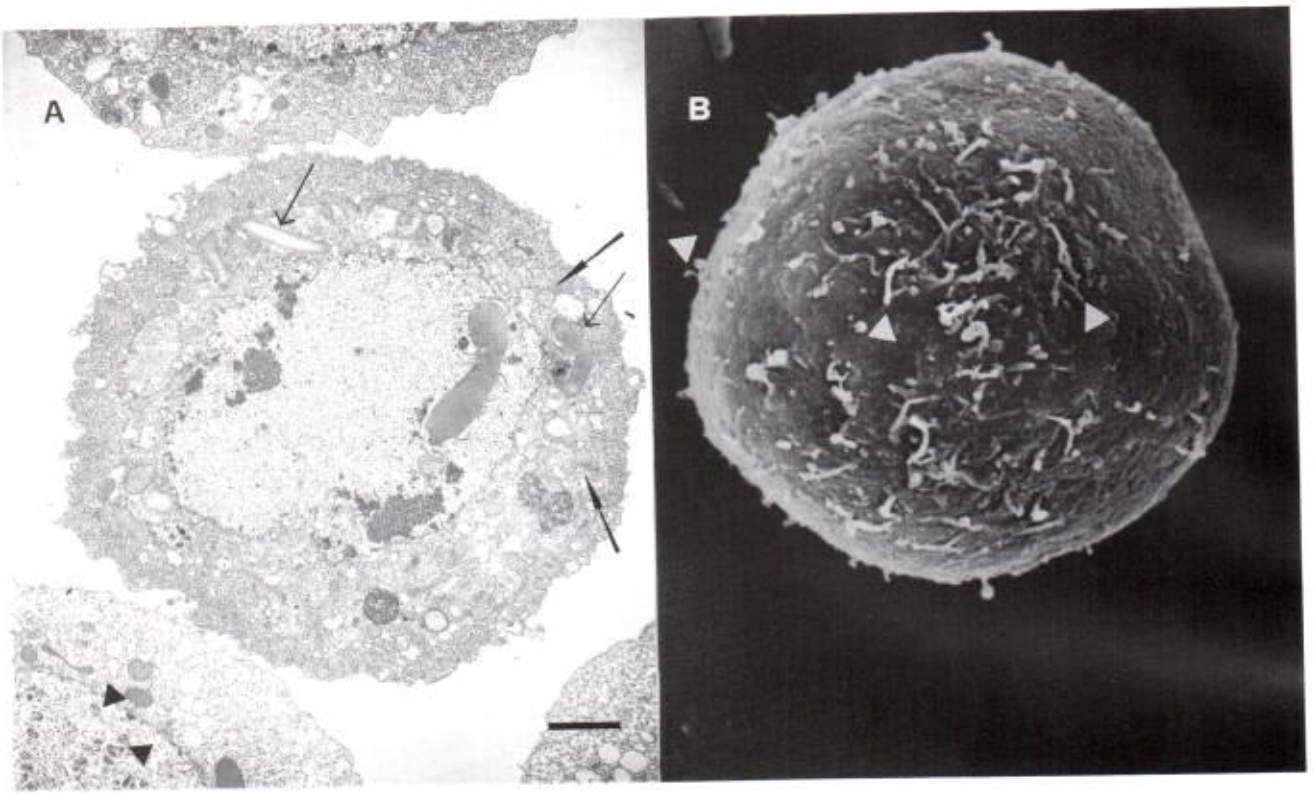

Fig. 3. a) Electron microscope analysis of Sf-9 cells 48 hrs after infection. Arrow: mitochondria, thin arrow: vesicles, Arrowhead: viral particles. Scale bar 0.3 μm. b) SEM analysis of Sf-9 cells 48 hrs after infection. Cell surface appears prevalently smooth with some microvilli (Arrowheads). Scale bar 0.3 μm.

cleolar organizer region (NOR) are observed. These modifications are probably related to the increased transcriptional activity of the infected cells. Scanning electron microscope (fig. 3b) analysis shows that the surface of these cells is mainly smooth, with the presence of some microvilli and large blebs, confirming the data observed by transmission electron microscopy (fig. 3a). Immunocytochemistry analysis (fig. 4b) shows that the cells contain a large amount of the synthesized TdT homogeneously distributed throughout the entire cytoplasm, while it is completely absent in the nucleus. These features are confirmed at higher magnification (fig. 5), which shows that the immunoprecipitate is not bound to any cytoplasmic structure. Initial stages of cell degeneration are observed 48 hours after infection.

DISCUSSION

Useful production of enzymatically active human terminal transferase has been obtained by the recombinant Baculovirus-infected Sf-9 insect cell system. The neosynthetized enzyme shows the same biochemical and molecular features as the TdT expressed by the thymocytes and human lymphoblastoid cells. Immunocytochemical analysis carried out by means of light and electron microscopy to identify terminal transferase showed that the enzyme is exclusively localized at the cytoplasmic level and that its synthesis begins soon after viral infection, increases with time and proceeds up to cell death. The negativity to immunocytochemical analysis of the Sf-9 nucleus suggests that the nucleus does not contain any specific structures able to bind the synthesized TdT which was produced and stored at the cytoplasmic level. The restriction of TdT exclusively at the cytoplasmic level also suggests that the nuclear membrane plays a possible functional role in the storage of TdT, behaving as a nonpermeable filter between cytoplasm and nucleus. Assuming that the mechanism of TdT synthesis in Baculovirus-infected cells is the same as that occurring in normally TdT-positive cells, it can be speculated that the transport system across the nuclear membrane and specific intracellular binding sites are involved in the intracellular movement of TdT. Sf-9 cells, which normally do not express any TdT, can acquire the capability of synthesizing the enzyme after viral infection, but probably lack the machinery for binding and subcellular redistribution of TdT, such as the nuclear matrix binding sites present in TdT-positive cells like thymocytes or

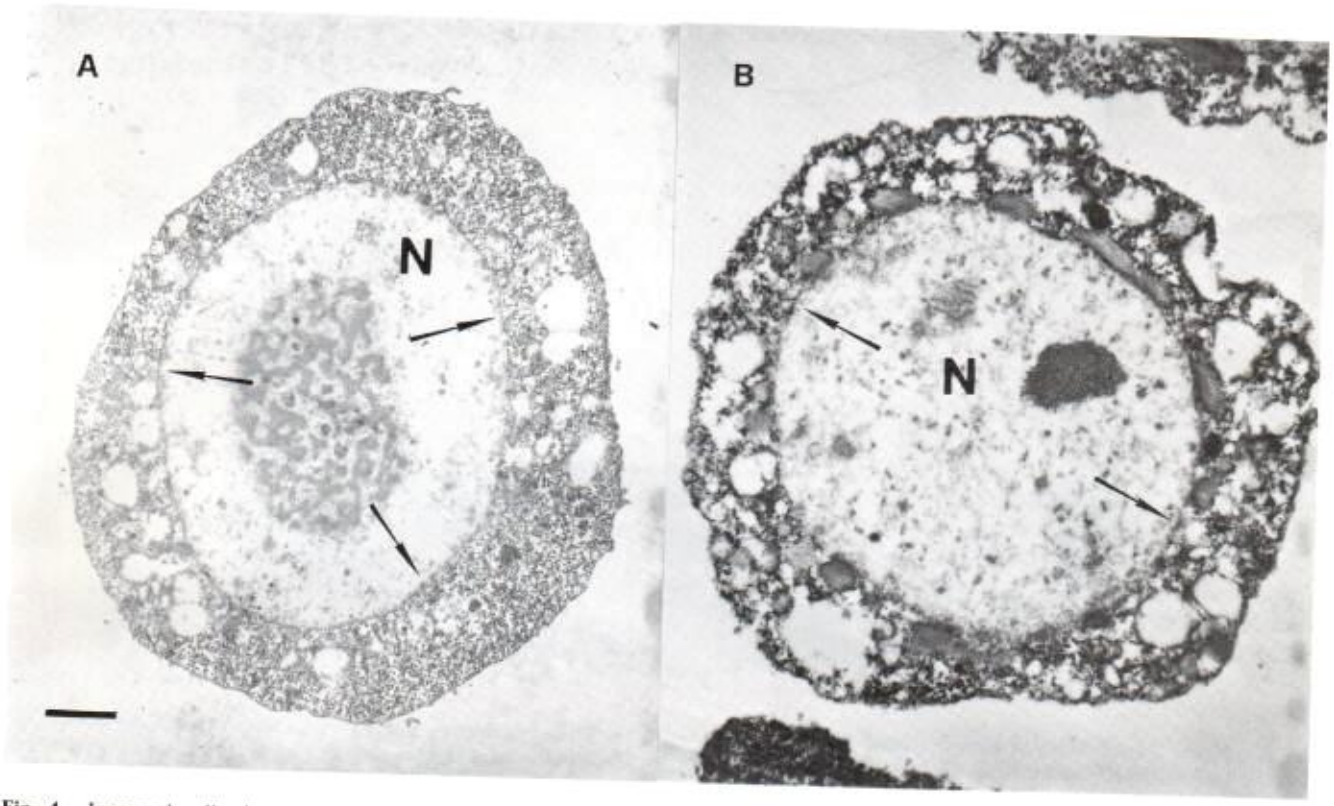

Fig. 4. Immunolocalization at cytoplasmic level of terminal transferase synthesized by the recombinant baculovirus system 24 (a) and 48 (b) hours after infection. Paraformaldehyde fixed/permeabilized cells. Rabbit anti-TdT as primary antibody, followed by peroxidase-conjugated goat anti-rabbit IgG. DAB reaction. It is possible to observe, at the cytoplasmic level, the different intensities of immunostaining reaction at various times of infection. Arrow: nuclear envelope; N: nucleus. Scale bar 0.3 μm.

lymphatic pre-T or pre-B cells. Therefore, this study indicates that TdT could gain access to the nucleus only when specific binding sites are provided. This condition seems to be restricted to the early stages of lymphocyte ontogeny and the unique so far reported cases of nuclear localization of TdT (7, 8).

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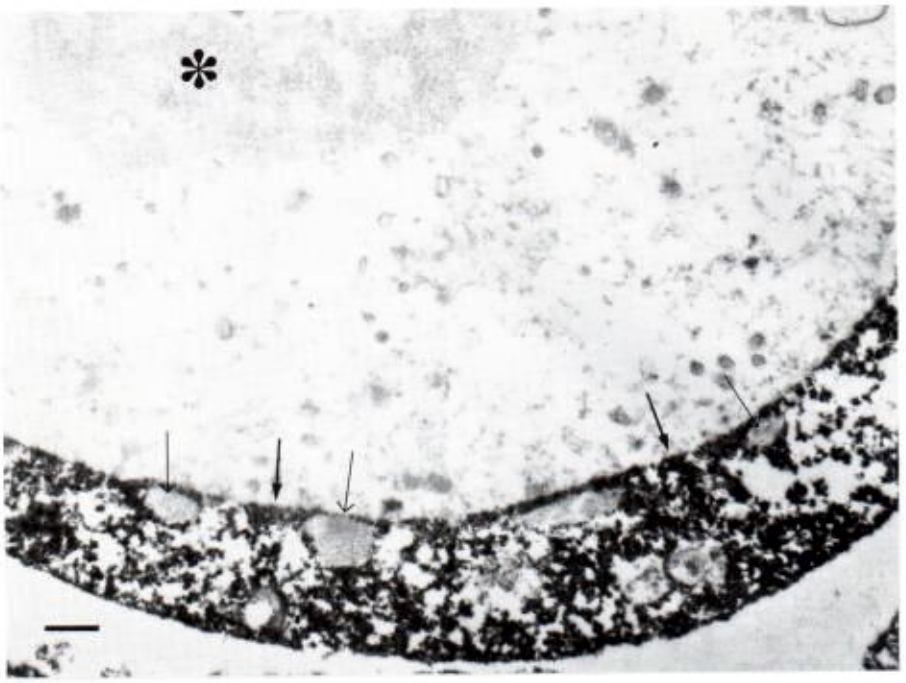

Fig. 5. Higher magnification of same preparation as observed in figure 4b. Arrow: nuclear membrane; thin arrow: vesicles; asterisk: NOR. Note dark immunoprecipitate localized only at cytoplasmic level. The nucleus and the NOR, as observed in Fig. 4a and b, are completely unstained. Scale bar 1 μm.

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