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The ER-TR4 monoclonal antibody recognizes murine thymic epithelial cells (Type 1) and inhibits their capacity to interact with immature thymocytes: immuno-electron microscopic and functional studies

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Abstract The thymic stroma is heterogeneous with regard to cellular morphology and cellular function. In this study, we employed the monoclonal antibody ER-TR4 to characterize stromal cells at the ultrastructural level. To identify the labelled cell type, we used two techniques: immunogold labelling on ultrathin frozen sections and immunoperoxidase staining on thick "vibratome" sections. ER-TR4 reacted with thymic Type 1 epithelial cells (according to our classification). A dense labelling appears in the cytoplasm of cortical cells using the two techniques. Immunogold labelling identified small cytoplasmic vesicles whereas the cytoplasm and the cell membrane seem to be labelled with the immunoperoxidase technique. ER-TR4 also identified isolated thymic nurse cells (TNC), and was observed in vitro to inhibit the capacity of some type 1 epithelial cells to establish interactions with immature thymocytes. This finding supports the hypothesis that the factor is involved in the formation of lymphoepithelial interactions within thymic nurse cells, and thus in the relations that immature thymocytes establish with the thymic microenvironment.

Introduction

The thymus plays a crucial role in the generation of T lmyphocytes (for review see Fowlkes and Pardoll 1989; Van Ewijk 1991). Differentiating thymocytes interact with stromal cells and, as a consequence, are triggered to proliferate and to mature. It is commonly thought that these events are mediated via direct cell-cell contact between lymphoid and stromal cells and/or via the secretion of short-range operating factors (Carding et al. 1991; Deman et al. 1992; Duijvestijn and Hoefsmit 1981; Kendall and Ritter 1988; Marrack et al. 1988, Ramsdell and Fowlkes 1990; Stutman 1978; Van Ewijk 1989).

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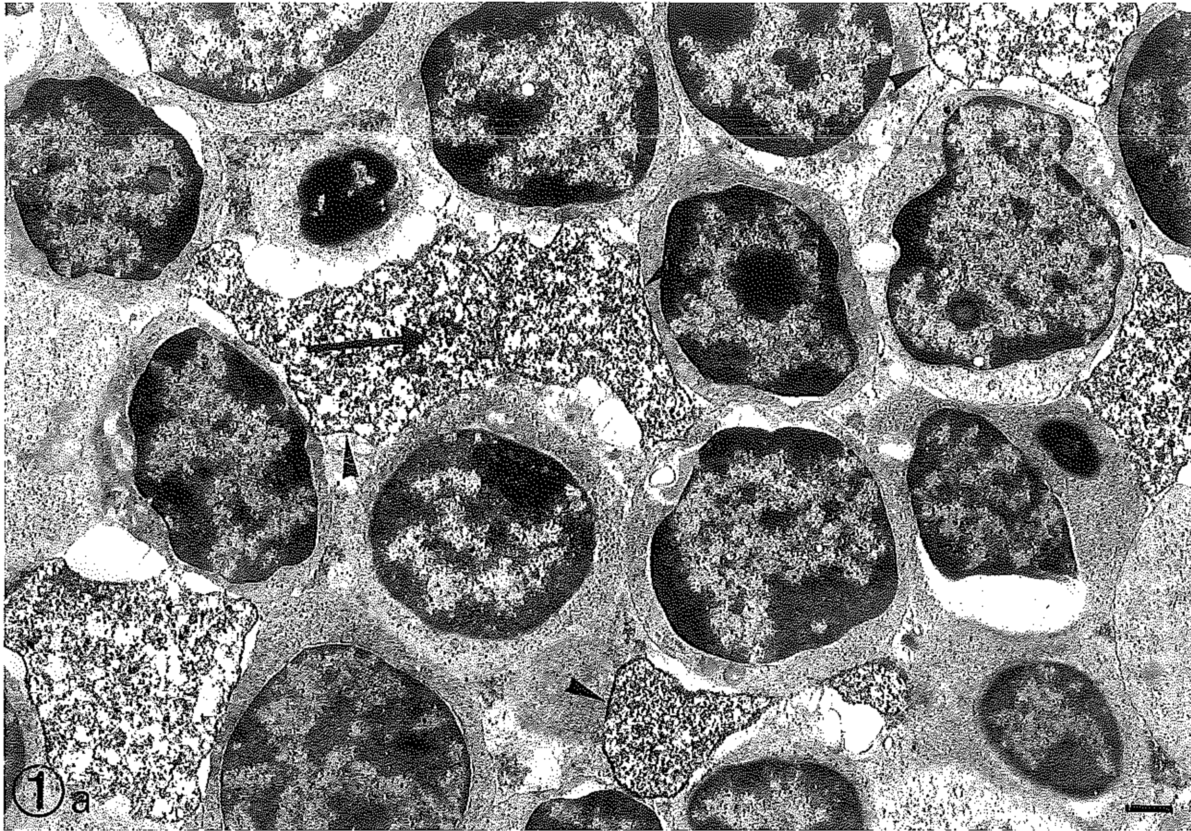
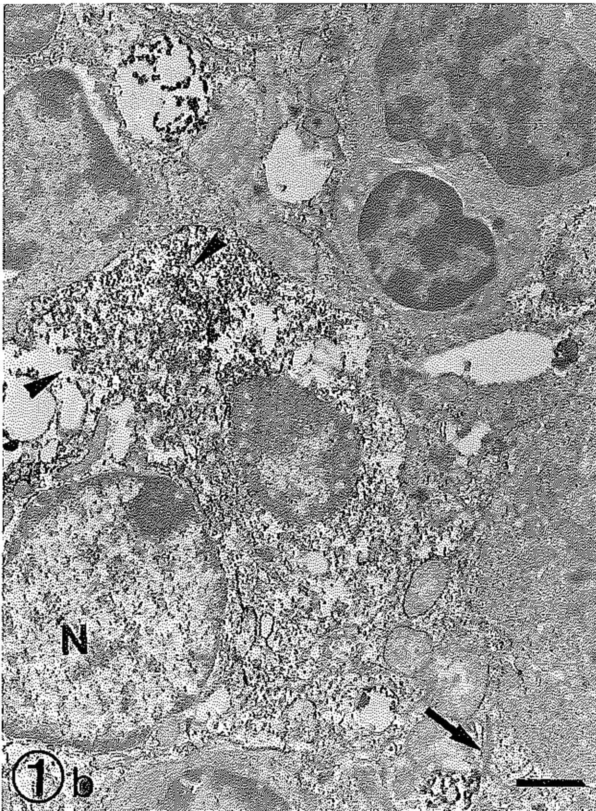
To understand the mechanisms underlying intrathymic lymphopoiesis, it is necessary to consider the complexity of the thymic microenvironment. Morphological studies have shown that the thymic stroma is composed of different cell types: (1) epithelial cells of which we have identified three types in the mouse thymus, and (2) two types of accessory cells originating in bone marrow (macrophages and interdigitating cells; for review see Nabarra and Andrianarison 1987, 1991).

In this report, we will consider the first type of epithelial cells (Type 1) observed in our classification, which corresponds to the "classical" epithelial cells described by numerous authors. Type 1 cells are observed in the cortex and a few in the medulla, whereas the two others (Type 2 and Type 3) are present only in the medulla (Nabarra and Andrianarison 1987). Type 1 epithelial cells are stellate in shape with numerous pseudopods extended between thymocytes forming a complex network. In addition to tonofilaments and desmosomes, these cells are characterized by the presence in the cytoplasm of a few "clear vacuoles" containing several dense granules.

A key question that arises is whether different stromal compartments control distinct events of intrathymic T cell differentiation. In a first approach, progress in this direction has been achieved by in vitro isolation of multicellular complexes from the thymus by enzymatic digestion and by sedimentation. Some of these complexes are identified as "thymic nurse cells" (TNC), i.e. epithelial cells enclosing intact lymphocytes (Andrews and Boyd 1985; Defresne et al. 1986, 1990; De Waal Malefijt et al. 1986; Hirokawa et al. 1986; Kyewski 1991; Kyewski and Kaplan 1982; Wekerle et al. 1980) and appear to play a role in T cell differentiation (De Waal Malefijt et al. 1986; Kyewski 1991). At the ultrastructural level, the thymic nurse cells show a characteristic cytoplasm of epithelial cells of Type 1 (De Waal Malefijt et al. 1986; Toussaint-Demylle et al. 1990).





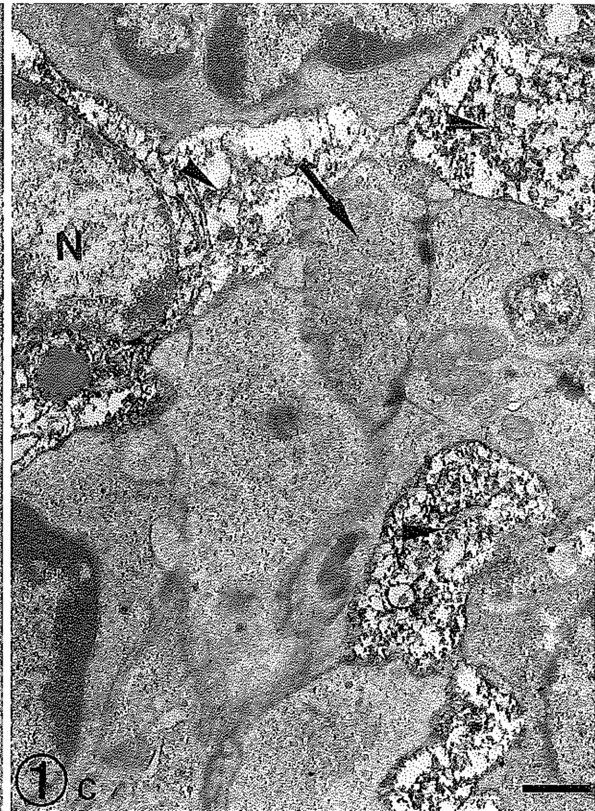


Fig. 1a–c Immunoperoxidase labelling on sections of murine thymus. **a** General aspect of the cortical zone showing labelling in the cytoplasm of epithelial cells (*arrow*). The slight retraction of the cells allows us better to observe the labelling on the cell surface membrane (*arrowheads*). Counterstained with uranyl acetate and lead citrate. ×6500; *bar*, 1 μm. **b** Staining of a Type 1 epithelial cell in the cortical zone (the *arrow* indicates tonofilaments and "clear vacuoles"). A great part of the cytoplasm is labelled by immunoperoxidase fine granulation (*arrowhead*). The plan of section passes by a nucleus indentation (*N*). Counterstained with uranyl acetate and lead citrate. ×9000; *bar*, 1 μm. **c** An epithelial cell is labelled (*arrowhead*) and the adjacent is not labelled (*arrow*). The processes of the labelled epithelial cell are extended between unlabelled cells. (*N* Nucleus). Counterstaining with uranyl acetate and lead citrate. ×9000; *bar*, 1 μm

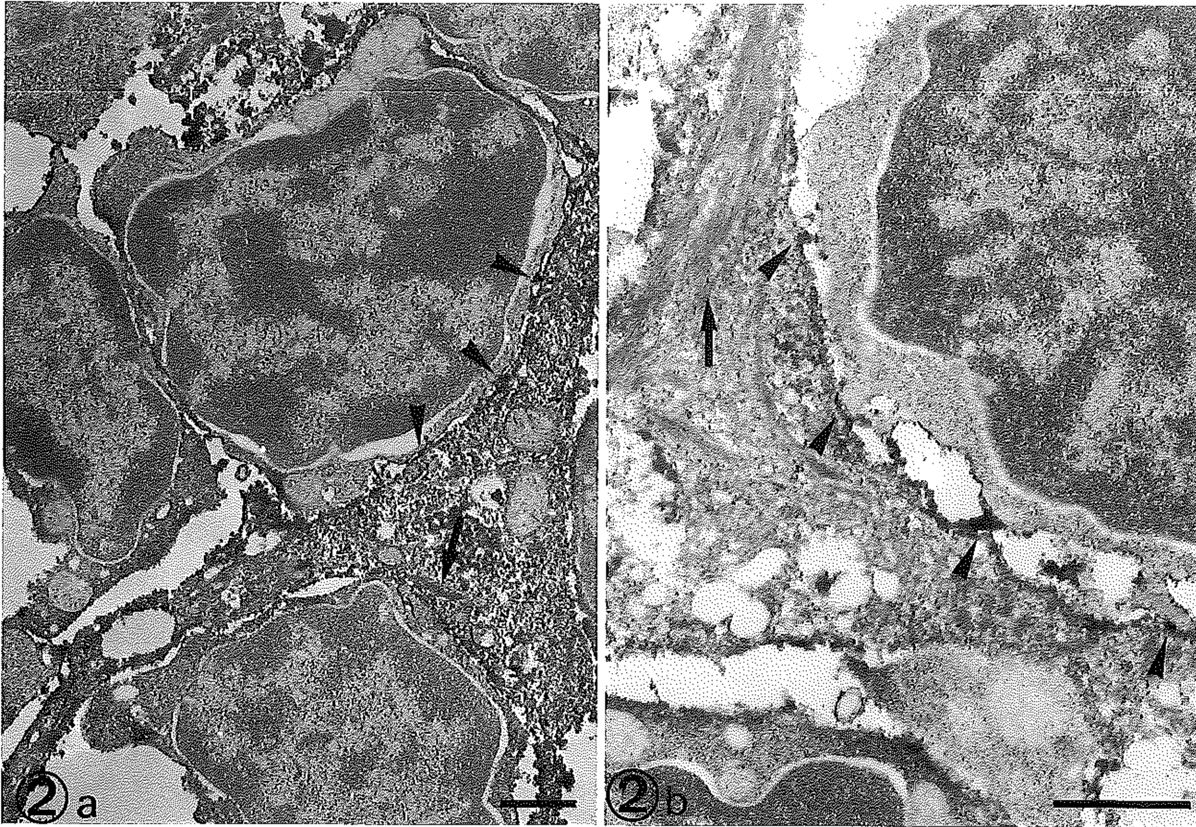


Fig. 2a, b Immunoperoxidase labelling between lymphocytes and epithelial cell surfaces. **a** In addition to the strong labelling within the cytoplasm of the epithelial cell (*arrow* indicates tonofilaments), patches of labelling were observed between lymphocytes and the epithelial cell surface (*arrowheads*). Section not counterstained. ×9500; *bar*, 1 μm. **b** High magnification of contact between labelled epithelial cell (*arrow* indicates tonofilaments) and lymphocyte. Labelling is observed along the cellular membranes (*arrowheads*). Section not counterstained. ×18000; *bar*, 1 μm

Another approach to study the complexity of the thymic stroma and to understand its role was to screen monoclonal antibodies against stromal cells on thymic frozen sections. At the light microscopic level, the use of numerous monoclonal antibodies led to the description of different thymic compartments (Rouse et al. 1988; Van Ewijk 1989, 1991; Van Vliet et al. 1984a). A few reports mention ultrastructural observations, which permit a true cellular identification of the cells labelled with different monoclonal antibodies (Farr et al. 1991; Haynes 1984; Van Ewijk et al. 1980b).

In this study we used, at the ultrastuctural level, the ER-TR4 monoclonal antibody described by Van Vliet et al. (1984a) to identify, at the light microscopic level, stromal cells in the thymic cortex and thymic nurse cells (Van Vliet et al. 1984a, b). We used two techniques of immunostaining at the ultrastructural level: (a) immunoperoxidase staining on "vibratome" thick sections and (b) immunogold labelling on ultrathin frozen sections. We show that ER-TR4 antibody reacts with Type 1 epithelial cells. In addition, we demonstrate that ER-TR4 inhibits in vitro the formation of lympho-epithelial complexes resulting from the interactions between TNC-derived epithelial cells and fetal thymocytes. The structures recognized by this monoclonal antibody thus are likely to be specialized epithelial cell molecules involved in the recognition process responsible for the formation of lymphoepithelial complexes controlling the early steps of intrathymic lymphopoiesis.

Materials and methods

Animals

Six- to eight-week-old C53 BL/6, DBA/2 and C3H/Hej mice (CNRS, Orléans, Iffa Credo, France and Erasmus University, Rotterdam, The Netherlands) were used for immunostaining. Four-week-old C57 BL/Ka mice (Stanford University, Calif., USA) were used for the in vitro experiments.

Monoclonal antibodies

The monoclonal antibodies (mAb) ER-TR4, ER-TR5, and ER-TR6 are rat IgM class antibodies previously described by Van Vliet et al. (1984). ER-TR4 reacts at the light microscopic level with cortical stromal cells and with isolated thymic nurse cells (Van Vliet et al. 1984a, b), ER-TR5 and ER-TR6 react with medullary stromal cells (Van Vliet et al. 1984a).

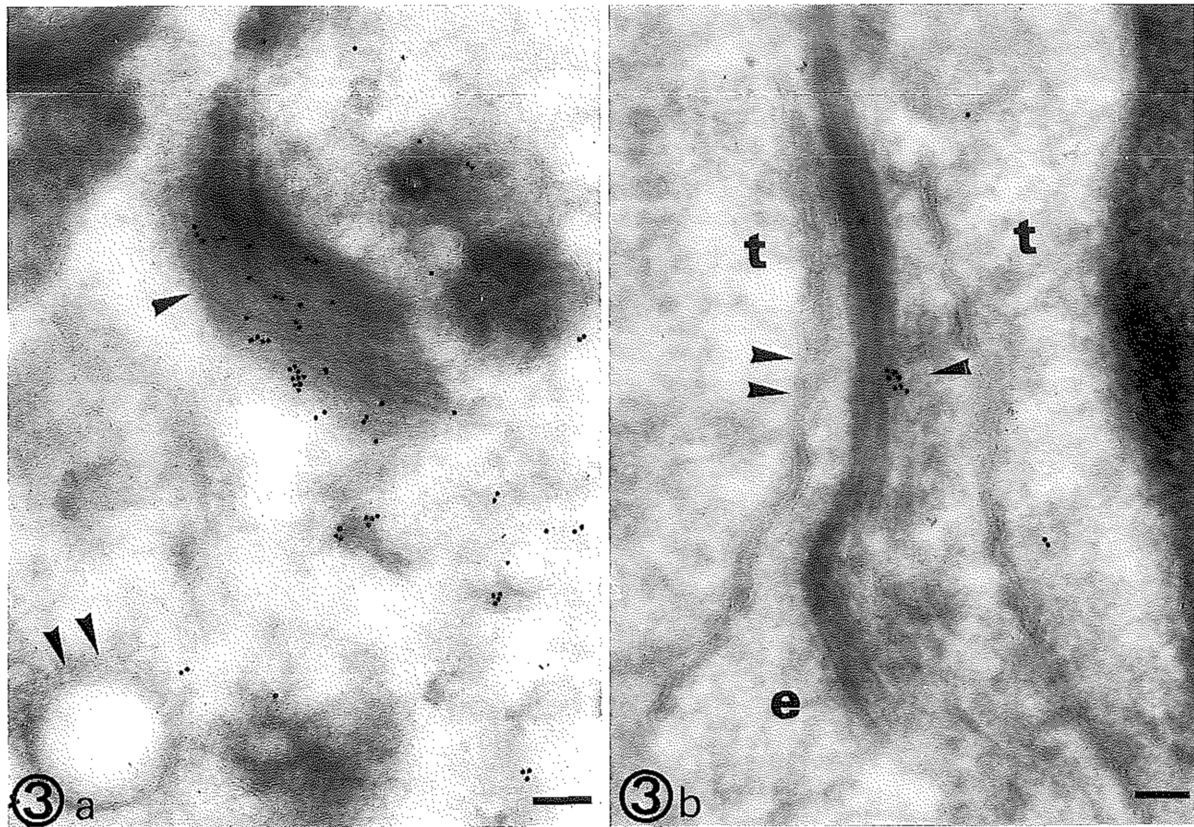


Fig. 3a, b Immunogold labelling of a cortical epithelial cell. *a* Labelling is observed on tonofilaments (*single arrowhead*, tonofilaments; *double arrowhead*, clear vacuole). Counterstaining with uranyl acetate. ×79750; *bar* 0.1 µm. **b** Process of the epithelial cell (*e*) extended between two thymocytes (*t*). The labelling is observed on a small vesicle (*arrowhead*) of the epithelial cell located in close contact with the tonofilament bundle. No cell surface staining is observed (*double arrowheads*). Counterstaining with uranyl acetate. ×70400; *bar*, 0.1 µm

Antisera and conjugates

For immunogold studies on thymic frozen sections, undiluted culture supernatant of ER-TR4 mAb was detected with a rabbit anti-rat immunoglobulin serum. This rabbit serum was detected with goat anti-rabbit immunoglobulin conjugated to 10-nm-diameter gold particles used at 1/20 dilution (Janssen Pharmaceutica, Beerse, Belgium). For immunoperoxidase labelling, the ER-TR4 mAb was detected with an anti-rat antibody (TEBU, France) conjugated with peroxidase and used at 1/50 dilution. To prevent non-specific binding, all reagents were optimally titered before use.

TNC isolation

TNC were isolated using a previously described procedure, including enzymatic dissociation of pooled thymuses and repeated sedimentation at 1 *g* (Houben-Defresne et al. 1982; Wekerle et al. 1980).

Preparation for immunoperoxidase technique

Small fragments of thymic tissues were fixed for 1 h at 4° C in 4% paraformaldehyde containing 0.1%, glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.4) and then cut into sections of 30–40 µm in thickness with a Vibratome (Balzers, Meudon, France). After overnight washing in 0.1 *M* cacodylate buffer, thymic sections were incubated with the first monoclonal antibody (ER-TR4) used as undiluted hybridoma supernatant with shaking, for 1 h at room temperature, followed by an anti-rat antibody conjugated with peroxidase. After several washings in buffer, sections were post-fixed lightly (for 15 min) with a 1% glutaraldehyde solution. After overnight washing in buffer, visualization was performed with 3,3'-diaminobenzidine tetrahydrochloride; sections were post-fixed in 1% osmium tetroxide, dehydrated in a graded series of alcohol and embedded in Epon. Ultrathin sections were made with a diamond knife and stained with uranyl acetate and lead citrate or not stained. Sections were examined with an EM 300 Philips electron microscope.

After isolation, thymic nurse cells (TNC) were fixed for 1 h in 4% paraformaldehyde containing 0.1% glutaraldehyde, in 0.1 *M* cacodylate buffer. After overnight washing, the incubation procedure used was similar to the procedure described above for thymus sections. Controls included (1) incubation with second step reagent only, and (2) normal rat serum instead of the specific antibodies.

Preparation for immunogold staining on ultrathin frozen sections

Mice injected with heparin were anaesthesized with avertin and perfused with phosphate-buffered saline (PBS) containing 0.1% Procaine/HCl, followed by perfusion with 0.1% glutaraldehyde and 2% paraformaldehyde in phosphate buffer (pH 7.4) for 45 min. Perfusion as total body perfusion was carried out via the heart as earlier described (Van Ewijk et al. 1980a). After perfusion, thymuses were excised, sliced into cubes of 1.2 mm of side and rinsed for 20 min in PBS containing 1.5 g glycine/l, in order

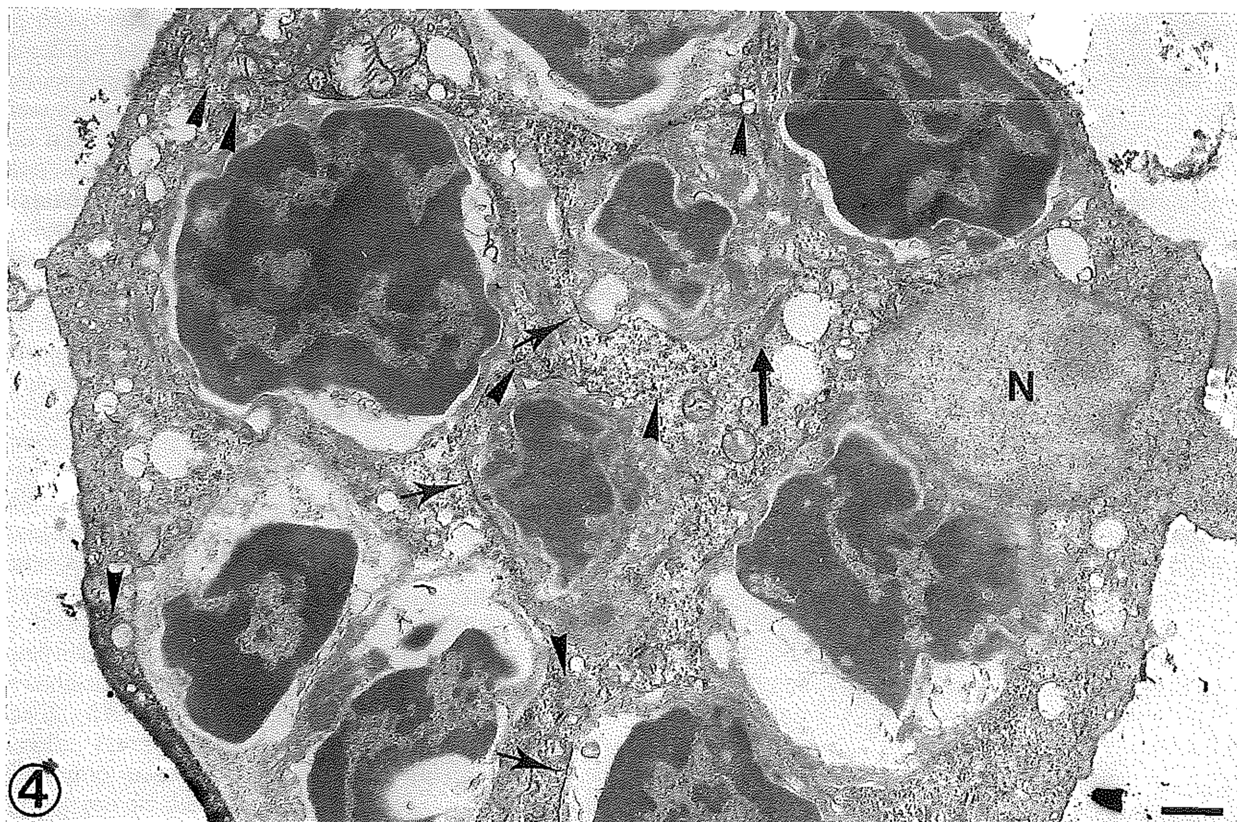


Fig. 4 Immunoperoxidase staining of an isolated thymic nurse cell (TNC) formation: the labelling (*arrowhead*) is observed on several parts of the cytoplasm of the epithelial cell (*arrow* indicates tonofilaments; *N* nucleus) as well as on a few parts of the cell surface membrane (*decorated arrowhead*) whereas the engulfed lymphocytes are not labelled. Counterstaining with uranyl acetate and lead citrate. ×8500; *bar*, 1 μm

to block free radicals and aldehyde groups and treated according to Tokuyasu (1973, 1984). Freezing of the specimens was performed by rapid immersion in liquid nitrogen. Ultracryotomy and immunostaining were carried out as described previously (Tokuyasu 1973, 1984). The control specimens consisted of ultrathin sections treated either with the second and the third antibody or with the third antibody alone. The sections were collected on grids, stained with uranyl acetate, and embedded in a film of 1.5% methylcellulose (400 cP) (Sigma, Germany) before examination.

In vitro TNC formation assay

To analyse the capacity of epithelial nurse cells to form lympho epithelial complexes, we used a previously described in vitro assay (Defresne et al. 1990). Briefly, isolated TNC were cultured for 5 days in RPMI 1640 (Gibco, Belgium) culture medium supplemented with 10% fetal calf serum; lymphocytes released into the medium were removed and the epithelial cells trypsinized and incubated with 16- to 17-day-old fetal thymocytes in inverted Terasaki plates (Nunc, Denmark). After 6 h of incubation, the proportion of epithelial cells having reformed lymphoepithelial complexes similar to freshly isolated TNC, was established by inverted phase contrast microscopy. The blocking property of ER-TR4 antibodies during recolonization was tested by incubating the cells with undiluted hybridoma supernatant. As controls, the cells were treated by ER-TR5 and ER-TR6 hybridoma supernatant; these antibodies react only with medullary stroma cells (Van Vliet et al. 1984a).

Results

Immunoelectron microscopic characterization of ER-TR4-labelled cells

At the light microscopic level, frozen thymus sections incubated with ER-TR4 antibodies show a reticular pattern in the thymic cortex, whereas the medulla was immunonegative (Van Vliet et al. 1984a).

Electron microscopic observations with immunoperoxidase labelling

The ER-TR4 label appeared on Type 1 epithelial cells (Fig. 1a), which are located principally in the cortex and in small numbers in the medulla: they are identified on unstained and stained sections by the presence of tonofilaments and of clear vacuoles containing electron dense granules (Nabarra and Andrianarison 1987). Other epithelial cell types (such as Type 2 and 3) only present in the medulla were not labelled (data not shown).

The great majority of Type 1 epithelial cells labelled with ER-TR4 were located in the cortex. Labelling with ER-TR4 was observed in different parts of the epithelial cells, occurring mostly in the cytoplasm and appearing as coarse, dense, granular material uniformly distributed throughout the cytoplasm or accumulated in cytoplasmic patches (Fig. 1a–c). The cell surface membrane was sometimes also labelled in more or less electron-dense

detected by ER-TR4 could thus be involved in the recognition process leading to the formation of TNC. This hypothesis is supported by the fact that the antigens recognized by these antibodies are located in the areas where lymphocytes are in close contact with epithelial cells. The nature of the molecules recognized by ER-TR4 antibodies remains to be determined.We have previously demonstrated (Defresne et al. 1990) that Ia antigens are also involved in interactions between epithelial cells and immature thymocytes. However, ER-TR4 does not recognize Ia antigens since the distribution of molecules labelled by ER-TR4 is different from that of Ia antigens, which are expressed not only on epithelial cells but also on some macrophages and on interdigitating cells (Kyewski et al. 1982; Van Ewijk 1991). On the other hand, it was shown that adhesion molecules, such as LFA-1 and ICAM-1, also play a role in the interactions between epithelial cells and thymocytes (Singer 1990; Singer et al. 1990). Whether or not ER-TR4 recognizes adhesion molecules cannot be determined from our experiments.

In conclusion, we have shown here that an antigen expressed by Type I cortical epithelial cells of murine thymus appears at the ultrastructural level in the cytoplasm and possibly at the cell surface. The functional role of this molecule, which is involved in lymphostromal interactions between thymic Type 1 epithelial cells and immature thymocytes, remains to be elucidated.

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