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IN SITU CHARACTERIZATION IN FREEZE-FRACTURED MOUSE THYMUSES OF LYMPHOEPITHELIAL COMPLEXES ULTRASTRUCTURALLY SIMILAR TO ISOLATED THYMIC NURSE CELLS

Key words: Thymic nurse cells, lymphoepithelial complexes, freeze-fracture, electron microscopy

ABSTRACT. Scanning and transmission electron microscopy of the cracked surfaces of cryofractured pre-fixed C57BL/Ka mouse thymus reveals the existence of cell complexes, distinct from the surrounding cell organization, in which groups of lymphocytes are delimited by large cytoplasmic sheets or envelopes. These complexes, located in the subcapsular and cortical regions, display morphological features similar to that of the thymic nurse cells (TNCs), which can be isolated from the mouse or human thymus enzymatically dissociated. They can be considered as dynamic systems able to modify their three-dimensional organization, namely with regard to intrathymic cellular traffic involved in T-lymphocyte maturation.

Introduction

The intrathymic events of T-cell differentiation occur under the control of the so-called thymic microenvironment. Seen from this aspect, the maturation of T-lymphocytes requires interactions with non-lymphoid stromal cells. Among these cells, thymic nurse cells (TNCs), which were first isolated by Wekerle and Ketelsen (1980a) from mouse thymus after enzymatic dissociation, are thought to provide some of the microenvironmental factors necessary to lymphocyte proliferation and differentiation (Wekerle *et al.*, 1980; Kyewsky and Kaplan, 1982). These large lympho-epithelial complexes show a unique spatial organization resulting from the close association between intact, actively dividing thymocytes and large cytoplasmic processes of a surrounding epithelial cell.

A critical question is to know whether the TNCs isolated *in vitro* have a counterpart in the thymus *in situ*. In the human thymus, Ritter *et al.* (1981) recognized the presence of TNCs on frozen sections and more recently Wijngaert *et al.* (1983) described on thin sections, large *in situ* lymphoepithelial complexes morphologically comparable to isolated TNCs. In the mouse thymus, however, no ultrastructural observation has hitherto formally detect *in situ* the presence of TNCs as three-dimensional structures although some histological surface labellings *in situ* (Andrews and Boyd, 1985) and several experimental data (Wekerle *et al.*, 1980; Kyewsky and Kaplan, 1982) seem to indicate that the TNCs are not *in vitro* artefacts. In the present work we have applied the cryofracture method developed by Humphreys *et al.* (1974) and Tokugana *et al.* (1974) combined with scanning (SEM) and transmission (TEM) electron microscopy to study the

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three-dimensional configuration *in situ* of the mouse thymus. In fact, this method has previously made possible the direct observation of the internal spatial organization of different organs, such as the mammal kidney (Fujita *et al.*, 1976; Hay and Evan, 1979) the adrenal cortex (Motta *et al.*, 1979) and the human placenta (Castellucci *et al.*, 1980).

Material and Methods

Preliminary observations with the SEM have shown that the cracked surfaces of thymus slices previously fixed according to routine procedures were inadequate to visualize any three-dimensional organization of the tissue. These procedures produced too flat fractures and yielded no more information than ultrathin sections. As described below, the stereoscopical observation of the fractured surfaces was significantly improved by the use of thiocarbohydrazide (TCH) and diluted osmium solutions as fixing agents.

(a) *TCH-cryofracture method*

Small pieces of thymus tissues were excised from normal 30-day-old C57BL/Ka mice. They were directly fixed by immersion for 24 hr in 4% glutaraldehyde–0·1 M-cacodylate buffered solution (pH 7·4), washed in the same buffer for 1 hr and post-fixed in buffered 1% OsO_4 for 2 hr at 4°C. After washing in distilled water, the specimens were treated twice by 1% TCH and 1% OsO_4 aqueous solutions according to Malick *et al.* (1975) and dehydrated in graded ethanol series. The samples were then transferred in Parafilm cylinders filled with absolute ethanol, frozen in liquid nitrogen (Humphreys *et al.*, 1974; Tokugana *et al.*, 1974) and cross-fractured in slices with the use of a frozen razor blade and a hammer. The slices were immediately returned to cooled absolute ethanol before critical-point drying in liquid CO_2 and examination in an Etec-Autoscan scanning electron microscope.

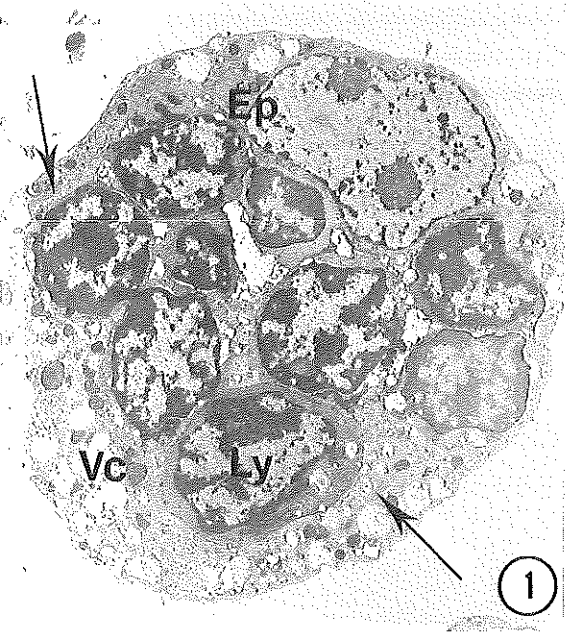
The TCH procedure prevents the metal coating of biological specimens before examination with the SEM and the obliteration of small and intricate surface details. We have also noticed that TCH treatment makes the material more brittle. Under these conditions, the fracture planes have the propensity for splitting along natural interfaces, i.e. plasma membranes; hence the probability of vizualizing the cell complexes associated with membrane processes is greatly enhanced. On the other hand, absence of an evaporated metal layer allows any part of the SEM specimen to be easily removed and processed for TEM. With this end in view, fracture areas exhibiting presumable lympho-epithelial processes were marked, carefully excised from the SEM specimens and embedded in Epon 812 with

Fig. 1. Thin section of a typical enzymatically isolated TNC. The epithelial cell (Ep) displays a large nucleus with two reticular nucleoli. The cytoplasmic processes containing tonofilament bundles (arrows) and numerous vacuoles (Vc), engulf small and blast-like lymphocytes (Ly). ×5000.

Figs 2, 3. SEM micrographs showing two extreme morphological aspects of isolated TNCs. They look like closed bag-shaped complexes limited by a poorly ruffled envelope (Fig. 2) or like a largely open multiperforated system (Fig. 3). Fig. 2, ×3000; Fig. 3, ×2000.

Fig. 4. SEM. TCH-cryofracture method. Subcapsular lymphocyte association covered by a perforated envelope. Lymphocytes can be viewed through envelope perforations (white arrow). ×1000.

Fig. 5. TEM. TCH-cryofracture method. Section perpendicularly orientated to the fracture plane of a thymus fragment removed from SEM specimen. A narrow cytoplasmic extension (arrow) of the epithelial cell (Ep) runs parallel to the cleavage plane (see also Figs 6 and 7). ×5000.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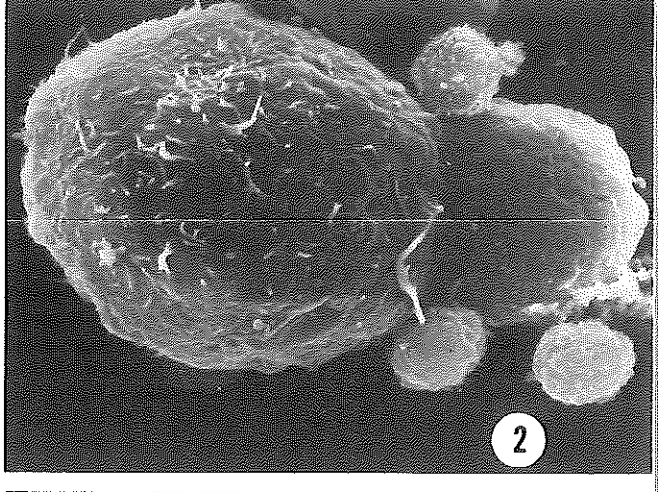
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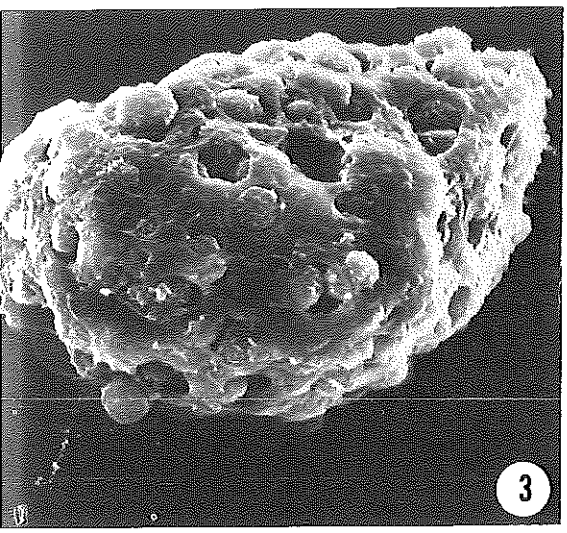
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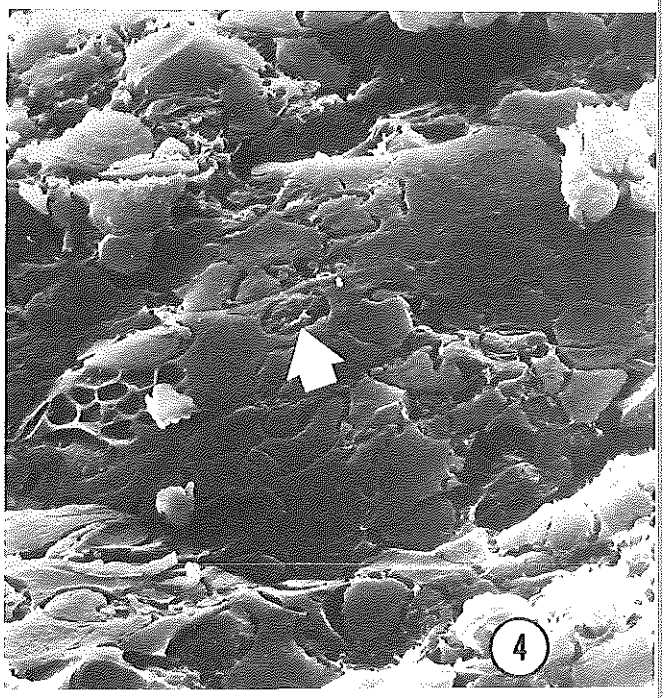
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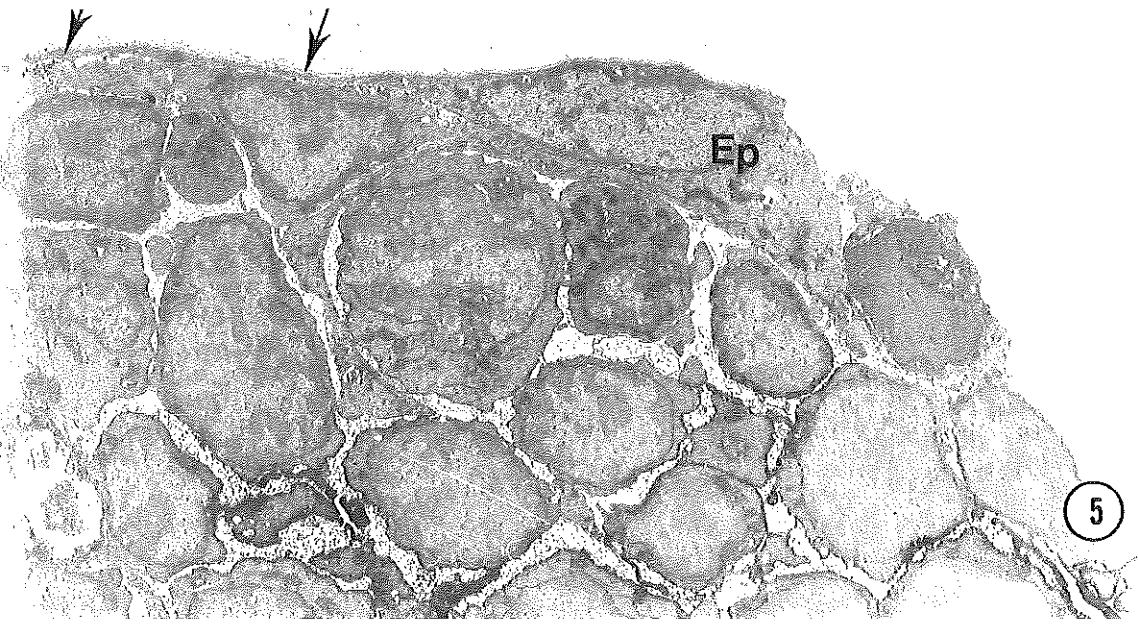
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the use of flat silicone rubber mould in order to facilitate their orientation for sectioning.

(b) *Osmium/DMSO/osmium-cryofracture method*

Other tissue fragments cut off from the same thymuses as in (a) were directly fixed in 0·07 M phosphate buffered 1% OsO_4 solution (pH 7·4 for 2 hr at 4°C. After rinsing with buffered solutions, they were successively incubated in 15, 30 and 50% dimethyl sulphoxide (DMSO) solution for 30 min each (Tanaka and Kinose, 1981) and immersed in 50% DMSO filled Parafilm cylinders before fracturing as described above. The cracked pieces were returned in cold 50% DMSO before washing in phosphate-buffered solutions. Then, they were left for 72 hr at 4°C in a phosphate-buffered 0·1% OsO_4 solution and post-fixed for 1 hr in a 1% OsO_4 solution. After dehydration in graded series of ethanol and critical point drying, some specimens were coated with gold–palladium (Hummer II sputtering apparatus) before examination in the scanning electron microscope.

The incubation with hypotonic osmic solutions of cells previously fixed by a chemical treatment involves a selective maceration of the cell material. This procedure removes from the cracked surfaces the excess cytoplasmic matrices, which hide cell organelles (Tanaka and Kinose, 1981). Moreover, as it will be mentioned in our results, this procedure, by its macerating effect, individualizes in bold relief some cell associations that are normally deeply imbricated in the thymic tissue.

As in TCH-cryofractured material, parts of tissue showing these reliefs were removed from the SEM specimens and embedded for TEM.

(c) *Isolated TNCs complexes*

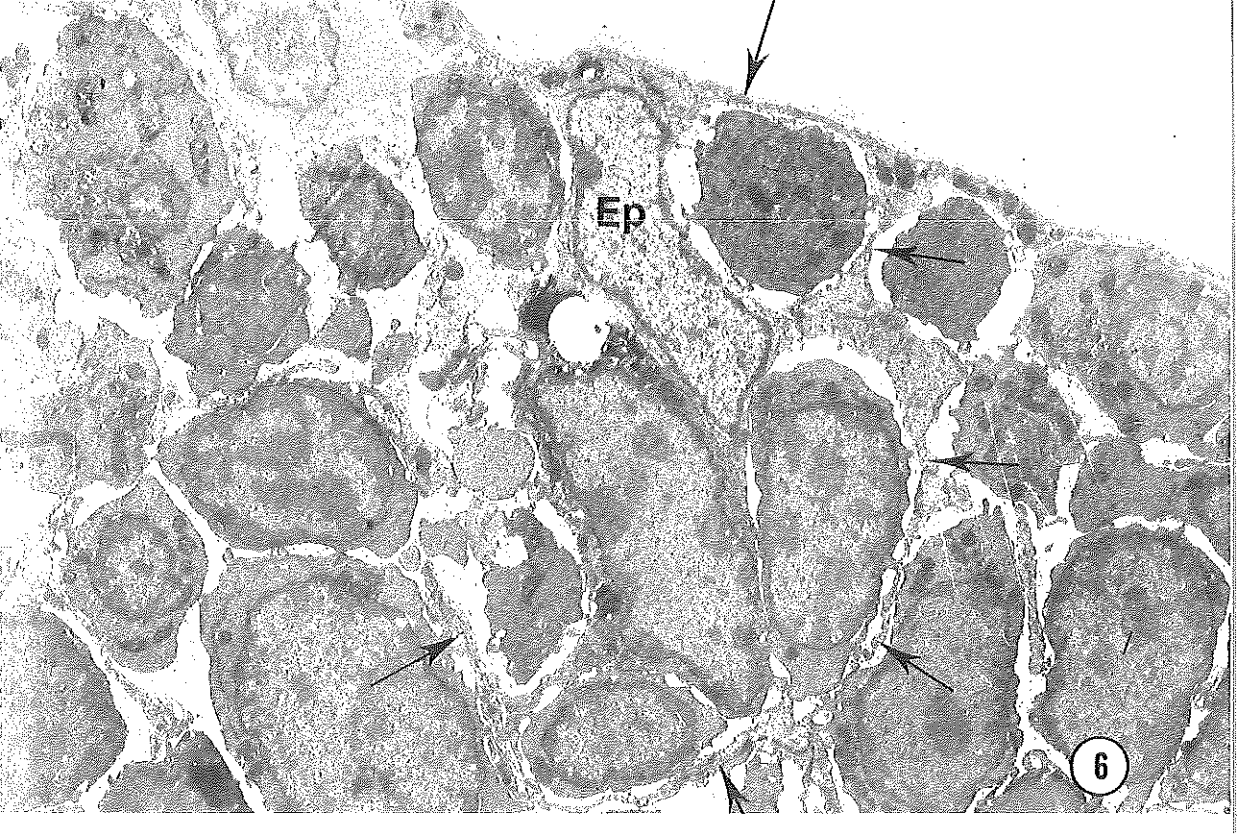
The TNCs isolation method, based on the original method of Wekerle and Ketelsen (1980), was described elsewhere (Houben-Defresne *et al.*, 1982). Briefly, thymuses were minced with scissors and washed for 10 min in PBS. After a digestion with collagenase the remaining fragments were incubated in the presence of dispase, DNase and collagenase. This latter enzyme solution was renewed two or three times and TNCs were isolated from the resulting cell suspensions by successive runs of 1 g sedimentation on 30% fetal calf serum. Aliquots of TNCs pellets were then routinely fixed in cacodylate buffered 2·5% glutaraldehyde and 1% OsO_4 solutions, dehydrated and either freeze-dried for SEM or processed for TEM.

Results

Since the first description of TNCs isolated from normal C57BL/Ka mouse thymus (Wekerle and Ketelsen, 1980), typical TNCs have been recognized in thin section as large round-shaped complexes formed by one epithelial cell engulfing numerous lymphocytes by long cytoplasmic processes (Fig. 1). Under the scanning electron microscope, which allows the visualization of large populations of TNCs, these complexes show a rather polymorphic profile; most of them are roundish to elongated in shape (Figs 2, 3). They are limited by a smooth, poorly ruffled envelope that often displays a few

Figs 6, 7. TEM. TCH-cryofracture method. Sections of thymus fragments removed from SEM specimens. The cytoplasmic extensions (arrows) of the epithelial cells (Ep) form a complicated network and delimit or isolate (Fig. 7) groups of lymphocytes. Fig. 6, ×5000, Fig. 7, ×5000.

Fig. 8. Thin section in the subcapsular zone of the normal thymus showing the epithelial cell (Ep) with a network of fine cytoplasmic processes (limits reinforced with black lines) that surround partially groups of lymphocytes. ×4500.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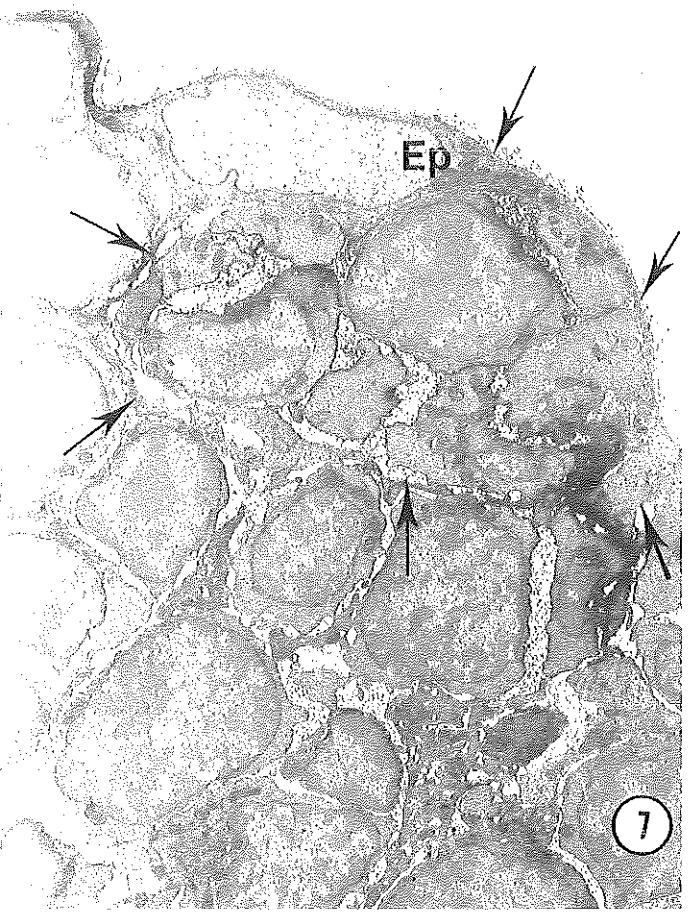
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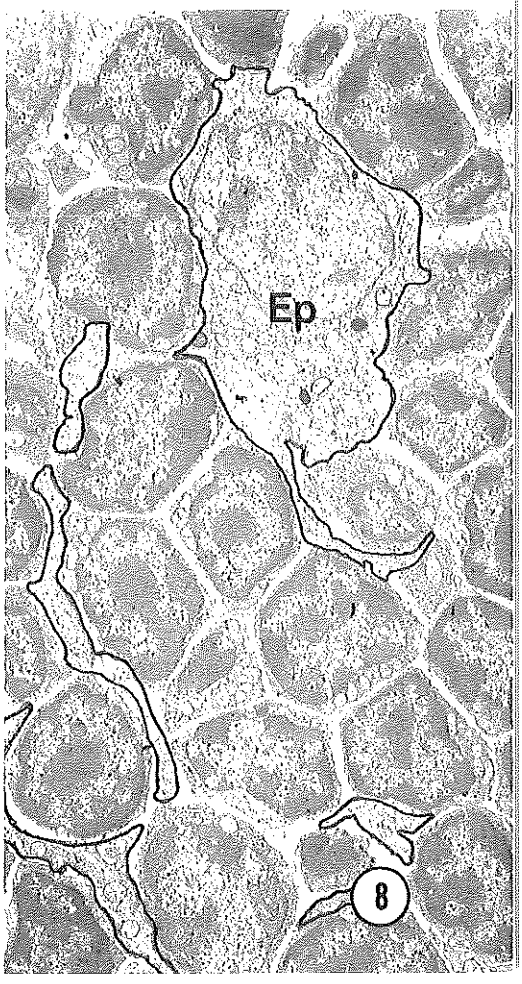
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perforations through which lymphocytes are recognizable. Occasionally, these perforations are very numerous (Fig. 3).

SEM observations of TCH-treated thymus fragments reveal that the cracked surfaces often coincide with cleavage planes that morphologically correspond to lamellar cytoplasmic sheets. These expansions appear as spreading smooth surfaces which delineate groups of lymphocytes in the subcapsular and cortical areas (Fig. 4). They show window-like holes in which lymphocytes are clearly recognizable as they are in isolated TNCs viewed by SEM. Sections perpendicularly orientated to these preferential cleavage planes show that the superficial sheets are long narrow cytoplasmic epithelial cell processes, which run parallel to the cleavage planes (Figs 5–7). Locally, these processes go on inside the tissue, so that they surround and engulf groups of lymphocytes. They quite often constitute a continuous envelope isolating some lymphocytes from the tissue as a whole (Fig. 7). Similar structural profiles consisting of cytoplasmic processes delineating partially groups of lymphocytes can be seen in thin sections of normal, non-dissociated thymuses (Fig. 8).

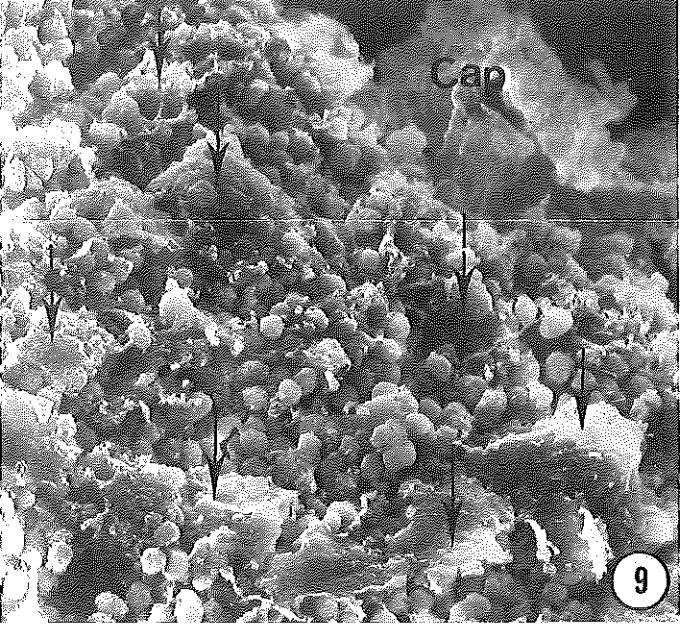
In comparison with fractured TCH-pretreated tissue, the osmium DMSO/osmium procedure induces some ultrastructural alterations, such as disruption and spongy aspects of the plasma membrane. However, mordancing and proteinic extraction from the cracked surfaces through hypotonic and dilute osmium solutions respectively lead to the appearance in bold reliefs of some structured cell associations in the vicinity of the thymus subcapsular region (Fig. 9). At higher magnification, these cell associations can be regarded as prominent roundish polymorphic lymphocyte clusters covered or enveloped by thin cytoplasmic sheets (Figs 10–12). As in isolated TNCs, the sheets display perforations through which lymphocytes can be observed (Figs 11, 12). Still more suggestive are the micrographs of these complexes when they are 'headed down' by a cracking plane. Under these conditions, they look like bag-shaped structures limited by a narrow cytoplasmic envelope, which extends from a large cell protruding inside the cavity filled with lymphocytes (Fig. 13). Moreover, even though the preservation conditions are not quite satisfactory, thin sections of such dome-like structures clearly show that the cytoplasmic envelopes can be undoubtedly related to epithelial cells in close association with groups of lymphocytes (Fig. 14).

Discussion

Previous direct morphological observations and monoclonal antibody labellings led to the conclusion that in the normal thymus there are various non-lymphoid cell populations that are thought to generate functionally distinct T-cell subsets (Zinkernagel,

Figs 9–14. Osmium/DMSO/osmium-cryofracture method. Subcapsular and proximal cortical zones of the thymus. Several cell complexes (arrows, Fig. 9) delimited by cytoplasmic sheets or envelopes are prominent in the plane of fracture. At higher magnification, the envelope shows holes (Fig 12) or protuberances (Figs 10, 11) corresponding to imprints of the subjacent lymphocytes, as it often does in isolated TNCs examined by SEM. When fractured ('headed-down'), these dome-like structures (Fig. 13) appear as bag-shaped complexes in which one can recognize the outer envelope (En) and lymphocytes (Ly); when cross-sectioned, they undoubtedly correspond (arrows) to epithelial cell prolongation (Fig. 14) in close association with groups of lymphocytes. Cap, capsular envelope. Fig. 9, ×750; Fig. 10, ×2000; Fig. 11, ×3000; Fig. 12, ×3000; Fig. 13, ×3000; Fig. 14, ×2000.

Fig. 15. Isolated TNC stained with FITC-conjugated anti-Thy-1 antibody viewed under normal white light conditions (left-hand micrograph). In fluorescence conditions, the same TNC (right-hand micrograph) shows a labelling of intra-TNC thymocytes. After isolation, TNCs were directly suspended for 30 min in FITC-conjugated anti-Thy-1 antibody optimally diluted in phosphate-buffered saline and fixed in a 2% formaldehyde solution before examination. ×200.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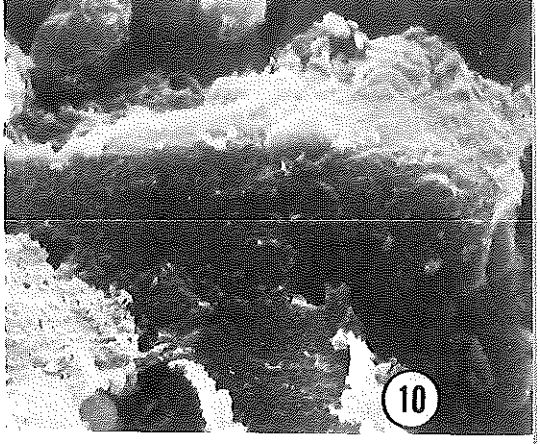
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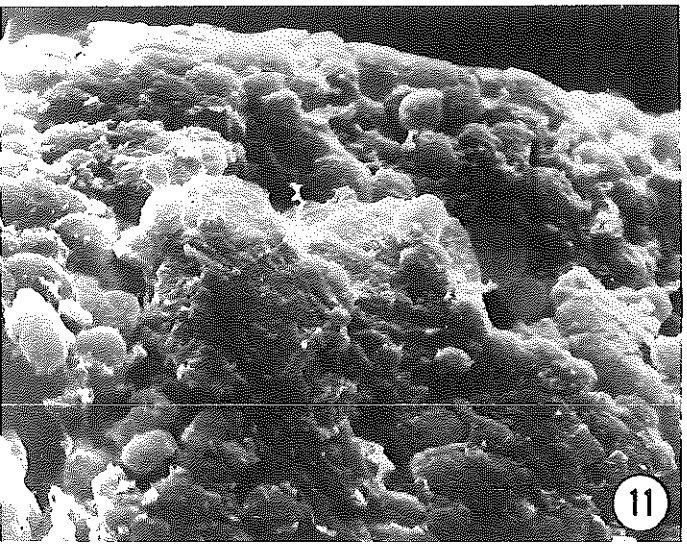
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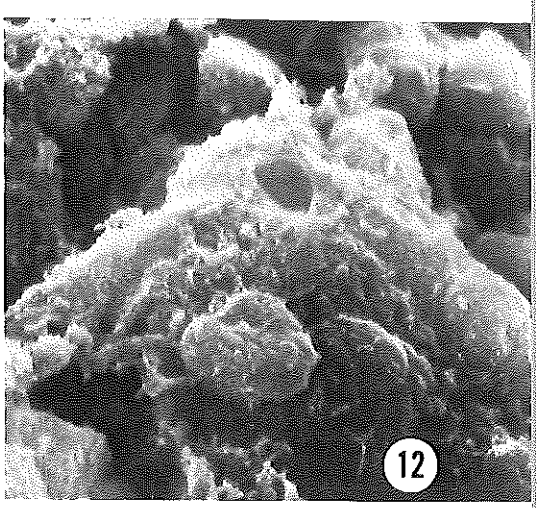
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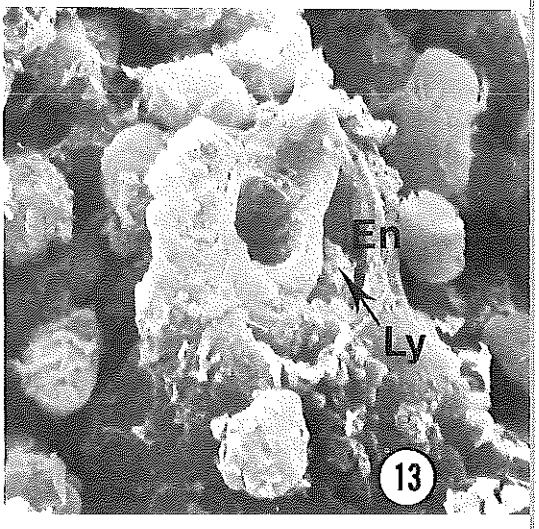
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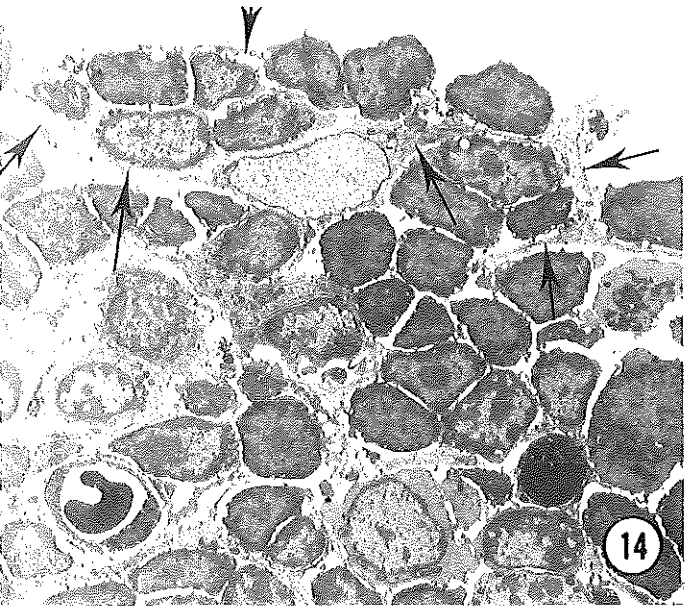
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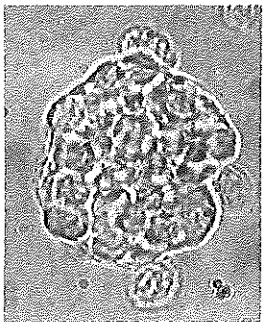

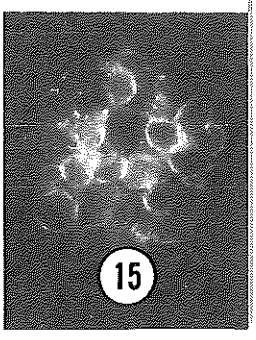
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1978; Longo and Schwartz, 1980; Scollay, 1983). Three main stromal elements can be recognized in the mouse thymus: macrophages, interdigitating dendritic cells and epithelial cells (Duijvestijn and Hoefsmit, 1981). The first two cell types are mainly located in the medulla and in the deep cortex and form with lymphocytes rosette-like associations after enzymatic dissociation of the thymus (Kyewski *et al.*, 1982). By contrast, some epithelial cells of the cortex, described as TNCs, establish a unique spatial arrangement by engulfing varying numbers, from 5 to 50 or 200 according to the authors, of apparently phenotypically immature thymocytes (Wekerle *et al.*, 1980; Houben-Defresne *et al.*, 1982; Kyewski and Kaplan, 1982).

However the real existence of TNCs *in vivo* as they structurally appear after dissociation remains hitherto questionable. Ultrathin sections of normal non-dissociated thymuses show at the level of subcapsular and cortical areas epithelial cytoplasmic networks delineating groups of lymphocytes (Fig. 8). Even if these structural profiles suggest the occurrence of compartments in the spatial distribution of lymphocyte populations, they do not formally demonstrate the 'morphological unity' of *in situ* lymphoepithelial complexes as enzymatically isolated TNCs do.

The present ultrastructural study combining SEM and TEM on cryofractured prefixed normal mouse thymuses demonstrate that TNCs are not artefacts that might result either from cell damage during enzyme dissociation or from endocytosis of lymphocytes during cell suspension preparation as suggested by some authors (Ritter *et al.*, 1981). The TCH cryofracture method denudes mechanically large cytoplasmic epithelial processes enveloping groups of lymphocytes, as they do in isolated TNCs. This propensity of the fractured plans for splitting following natural interfaces may be related to the ability to isolate TNCs routinely by mechanical dissociation only (Wekerle *et al.*, 1980), without any previous enzymatic treatment. On the other hand and in agreement with previous observations based on topic stainings with fluorescein isothiocyanate (FITC) (Kyewski and Kaplan, 1982; Houben-Defresne *et al.*, 1982; Andrews and Boyd, 1985), cryofractured thymuses either after TCH or DMSO-OsO_4 procedures reveal that these lympho-epithelial complexes are preferentially located in the subcapsular and in the outer cortical tissue. Moreover, there is an interesting parallelism between the *in situ* observation of lympho-epithelial complexes by the cryofracture methods and the capacity to isolate TNCs from similar thymuses. For example, in cryofractured thymic lymphomas induced by inoculation of the thymotropic leukemogenic radiation leukemia virus (RadLV), the typical three-dimensional organization of these complexes does not form any longer (Defresne *et al.*, unpublished). Now it has been shown that isolated TNCs are no longer recovered from such thymic lymphomas (Houben-Defresne and Boniver, 1983).

The final question is whether the morphological observations yield some indications on the function and the kinetics of the lympho-epithelial complexes. On the basis of histological *in situ* labelling with FITC-conjugated anti-Thy-1 and with polycationic dyes, it was shown recently (Andrews and Boyd, 1985) that TNCs reflect the existence of sealed structures, which are isolated from the general thymic milieu. However it seems unlikely that TNCs constantly form a totally secluded compartment, isolated from the thymus as a whole. On the contrary, our SEM observations either of isolated TNCs or of *in situ* lympho-epithelial complexes in cryofractured thymuses show that the outer envelope can display 'holes'; these could ensure direct communications between the TNC compartment and the extra-TNC milieu, i.e. the rest of the thymus, and hence provide channel-like structures for cellular traffic. This view is supported by experiments in which freshly isolated, not fixed TNCs were incubated with FITC conjugated anti-Thy-1 antibodies (Defresne *et al.*, unpublished): as shown in Fig. 15, the lymphocytes of a few TNCs are stained indicating that the antibody had passed through the epithelial envelope of the TNC, most likely through the 'holes' described in the present paper.

TCNs are commonly regarded as functional transit centres where lymphocytes find some microenvironmental conditions necessary to their maturation. Taking the present observations into consideration,

one can consider TNCs as dynamic systems, able to modify their three-dimensional configuration in the course of time, namely as regards the formation of 'openings', which allow lymphocytes to gain access or to leave the intra-TNC compartment. So the totally sealed TNCs could be regarded as a temporarily lympho-epithelial cell subset of the whole TNCs population corresponding to the time during which the enclosed structures could constitute, as suggested by Andrews and Boyd (1985), a barrier to diffusion ensuring a local high concentration of thymus hormones or other factors, shown to be produced in the outer cortex and thought to be involved in maturation processes (Savino *et al.*, 1982; Haynes *et al.*, 1984).

In conclusion, the present observations of cryofractured pre-fixed thymus demonstrate the presence of three-dimensional lympho-epithelial complexes in the subcapsular and cortical regions of the thymus. They display, in comparison with isolated TNCs, some morphological differences of minor importance (such as their polymorphic shape) that can be related to the isolation procedure. Our ultrastructural observations make us believe very strongly that the *in vivo* identified complexes are the *in situ* counterparts of the isolated TNCs. The available isolation method thus provides the investigator with a well-defined tool to study the role of specialized lympho-epithelial interactions in the intrathymic differentiation pathway.

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