Cell Tissue Res (1986) 244:673–679

DEFMSNE MP_21

Cell and Tissue Research

Multicellular complexes of thymocytes and different types of thymic stromal cells in the mouse*

Renata Brelińska[1], **Marie-Paule Houben-Defresne**[2], **and Jacques Boniver**[2]
Laboratory of Pathological Anatomy, University of Liege, Liege, Belgium

Summary. The isolation of multicellular complexes of thymocytes and different types of thymic stromal cells from the mouse thymus is described. Isolated complexes were examined by light microscopy. Stromal cells, binding thymocytes at their surface, were identified using electron microscopy, and three types of epithelial cells, macrophages and interdigitating-like cells (IDC-like cells) were distinguished from their morphological characteristics. The epithelial cell types correspond morphologically to epithelial cells present in situ in various thymic regions. The type of thymocyte-contact with epithelial cells, macrophages and IDC-like cells indicated that the formation of multicellular complexes is common.

Key words: Thymic cell complexes – Nonlymphoid thymic cells – Thymus – Mouse

The presence of heterogeneous populations of cells within the thymus, distinct with respect to origin, morphology and functions, has led to the model of cell-to-cell direct interactions between thymocytes and thymic stromal cells; these interactions might be responsible for the induction of thymocyte differentiation (Zinkernagel et al. 1978; Wekerle and Katelsen 1980; Farr and Nakane 1983). Some of the studies provide evidence for the specific binding of thymocytes to thymic stromal cells, although the physiological significance of this binding is not clear (Lipsky and Rosenthal 1973; Wekerle et al. 1980). Kyewski et al. (1982) have recently shown that thymic macrophages, dendritic cells and thymocytes can be isolated from disrupted mouse thymus as multicellular complexes. Other complexes, termed thymic nurse cells (TNC) have been obtained by Wekerle et al. (1980) from disrupted thymus. They are formed by cortical epithelial cells, each of which, in vitro, probably encloses within its projections a group of immature thymocytes.

Cell separation techniques used until now have been most useful for the isolation of separate cell populations (enriching them with respect to different subsets of thymus cells) or for the separation of defined thymocyte-stromal cell complexes (Loor 1979; Kyewski et al. 1982). In order to understand the relationship between thymocytes and thymic stromal cells, distributed within different topographical regions of thymus (i.e., cortex, cortico-medullary zone and medulla), we have experimentally separated them as multicellular complexes. In the present paper we report a separation of stable multicellular complexes, composed of different types of stromal cells and thymocytes, from mouse thymus.

Materials and methods

Animals

One hundred C57Bl/6 mice of either sex and about 2 months of age were used.

Obtaining thymocyte-stromal cell complexes

For each experiment, 20 thymuses were removed and placed in PBS (4° C) supplemented with 5% heat-inactivated (56° C, 30 min) fetal calf serum (FCS, Gibco Europe). After a single rinse, thymuses were minced with scissors into fine blocks. The tissue fragments were resuspended in PBS supplemented with 5% FCS and mixed gently with a magnetic stirrer at 0° C for 30 min. A cell suspension was decanted from these tissue fragments, filtered through Blutex nylon and collected as a single fraction. The remaining tissue fragments were subjected to enzymatic disssociation by incubating them with collagenase (2 mg/ml PBS, Boehringer Mannheim GmbH) with gentle mixing (magnetic stirrer), at 0° for 30 min. The incubation was repeated up to 5 times, until the thymic tissue was completely dissociated. Suspensions were filtered and collected as separate fractions and their complexes were purified, as described below. The steps of the isolation procedure are presented in Fig. 1.

Conditions of dissociation were determined in a preliminary series of experiments designed to estimate the type and number of cellular complexes, using media that con-

Send offprint requests to: Dr. R. Brelińska, Department of Histology and Embryology, Academy of Medicine, ul. Święcickiego 6, Pl-60-781 Poznań, Poland

[1] R.B. was a fellow of the University of Liege, on leave from the Department of Histology and Embryology, Academy of Medicine, Poznań, Poland

[2] Laboratory of Pathological Anatomy, University of Liege, B 23, Liege, Belgium. J.B. is Chercheur Qualifié du FNRS

* This work was supported in part by Grants from the Fonds de la Recherche Scientifique Médicale, the Centre Anticancereux près l'Université de Liège and by the Polish Academy of Science

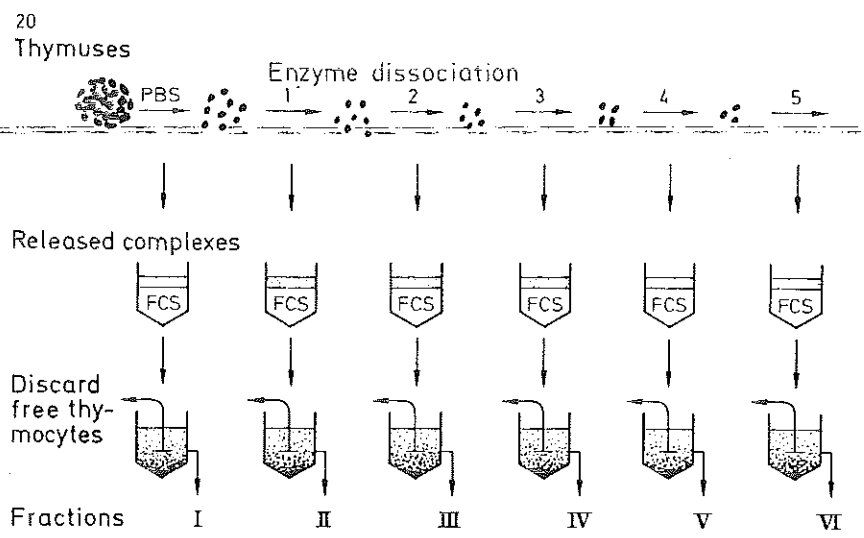


Fig. 1. The thymus dissociation procedure and purification of isolated multicellular complexes

tained collagenase, protease and deoxyribonuclease (Boehringer Mannheim GmbH, at 0.5, 4.8, 0.02 mg/1 ml PBS, respectively) and collagenase at 0.5 or 1 mg/1 ml PBS.

Purification of thymocyte-stromal cell complexes

The fractions obtained at each step of the enzyme dissociation (see above) were centrifuged at 100 g for 5 min at 4° C, resuspended in 5 ml PBS and further enriched with respect to cell complexes by 1-g sedimentation in FCS. The cell suspension containing approximately $2.5–3 \times 10^9$ cells was layered over 15 ml undiluted FCS in a conical plastic centrifuge tube. After sedimentation for 30 min, the top fraction containing the majority of lymphocytes was discarded. However, if the top fraction contained some cell complexes, the sedimentation was repeated. The FCS fraction (bottom) containing sedimenting cell complexes was centrifuged, the cells (approximately 1×10^9) were resuspended in 2 ml PBS and again layered over 10 ml FCS to purify the cell complexes further. The procedure of sedimentation in FCS was repeated several times and resulted in fractions that contained 50–80% cell complexes. Cell viability, estimated by the trypan-blue exclusion test, was approximately 98% in the crude cell suspension and 94% following sedimentation in FCS.

Light and electron microscopy of cell complexes

Cell recovery and enrichment of thymocyte-stromal cell complexes in the successive fractions obtained by the above procedure were estimated using light and electron microscopy. The total number of cell complexes and the number of free thymocytes in individual samples were determined using Thoma's hemocytometer. Cell fractions were fixed in 2.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4) postfixed in 1% OsO_4 in the same buffer, dehydrated and embedded in Epon. Randomly chosen blocks were serially sectioned (every 4 semithin sections followed by one ultrathin section). The cellular composition of complexes was estimated in semithin sections whereas ultrathin sections were used to examine the ultrastructure of complex-forming cells.

Results

The procedure for isolating cell complexes containing mal cells and thymocytes from the thymus of mouse consisted of several stages as described in Materials and methods. The first of them involved a 30-min incubation of thymus fragments in PBS with delicate mixing. It yielded on average 4.2 to 4.8×10^4 cell complexes per thymus. Continued incubation in PBS yielded no additional cell complexes. Sequential incubations of the tissue in a solution containing 2 mg collagenase per 1 ml PBS were more efficient. The distribution of complexes into individual fractions was significantly affected by the degree of tissue fragmentation and by the rate of mixing the tissue fragments. The most reproducible results were obtained when tissue fragments of dimensions $0.5 \times 1 \times 1$ mm were mixed at 60 rev./min. Results obtained under such conditions are presented in Table 1. In 5 consecutive experiments, the deviations in complex percentage in individual fractions did not exceed 17%. The average number of complexes per young adult thymus was about 40×10^4.

Light microscopy

Studies using light microscopy allowed the preliminary characterization of thymocyte-stromal cell complexes and the estimation of their content in sequential fractions. The cell complexes were composed of thymus stromal cells, each of which bound at least two and sometimes more than ten thymocytes.

Taking into account their morphological traits, three types of complexes were distinguished:

I. Complexes formed by large cells showing characteristic features of TNC, including the content of thymocytes. At their periphery, 2 to 10 thymocytes were seen.

II. Complexes formed by medium-sized or large stromal cells, oval in shape and sometimes of irregular outline. The cells showed no features typical of phagocytic cells. At their periphery, 2 to 6 thymocytes were seen.

III. Complexes in which thymocytes surrounded the central cell, thus making its identification impossible. Individual complexes of this type differed from one another in the

Table 1. Number of cell complexes in sequential isolation stages (per thymus). The yields represent a typical experiment

Fractions	Media	Number of complexes $\times 10^4$	Type of complexes					
			I		II		III	
			number $\times 10^3$	%	number $\times 10^3$	%	number $\times 10^3$	%
I	PBS	4.2	2.2	5.2	4.5	10.7	35.6	84.2
II	collagenase	5.2	4.6	8.9	0.3	0.6	47.2	90.5
III	collagenase	19.1	17.4	9.1	20.2	10.6	153.2	80.3
IV	collagenase	2.9	4.6	16.2	3.2	11.1	20.8	72.7
V	collagenase	6.7	10.2	15.1	32.2	48.0	24.8	36.9
VI	collagenase	0.9	0.8	8.7	3.5	36.3	5.3	55.0
Total number		39.1	3.9×10^4		6.4×10^4		28.7×10^4	

size of the central cell and number of thymocytes bound. 10 to 30 thymocytes were seen around the central cell.

Some complexes tended to form large cell accumulations; these were interpreted as resulting from clustering of a few complexes of stromal cells and thymocytes. The numbers of complexes distinguished within individual fractions are presented in Table 1; as can be seen, individual fractions differ in the number and type of the observed complexes.

Electron microscopy

For improved identification, cell complexes of individual fractions were analyzed in serial semithin and ultrathin sections. The complexes were formed around either thymic epithelial cells or stromal cells of mesenchymal origin.

The epithelial cells forming complexes with thymocytes were heterogeneous in morphology and could be grouped into three distinct types.

Epithelial cells type 1 involved large cells, containing numerous caveolae that surrounded thymocytes (Fig. 2). Their cytoplasm contained electron-lucent vacuoles with a small amount of electron-dense material and bands of microfilaments. Thymocytes contacted their surface by short projections or by forming wider, planar contacts. This cell type corresponded to type-I complexes distinguished by light microscopy and thus to TNC.

Epithelial cells type 2 included large cells with considerable amounts of cytoplasm and an eccentrically located nucleus (Figs. 2, 3). Vacuoles were noted at one of the cell poles. The cytoplasm contained numerous cisterns of smooth endoplasmic reticulum and individual bundles of microfilaments. In some cells, multiple bundles of microfilaments were associated with an amorphous electron-dense material. Thymocytes contacted the cell membrane of epithelial cells type 2 by short projections or by adhering over a wider area (Fig. 4). The epithelial cells were present in complexes identified as complexes type II using the light microscopy.

Epithelial cells type 3 included medium-sized cells containing a frequently irregular, peripherally-located nucleus (Figs. 3, 5, 6). Some of the cells showed a relatively regular outline whereas in other cells the cell membrane formed a multiplicity of densely clustered projections that contacted other stromal cells or thymocytes. The cells contained vacuoles and exhibited typical microvilli or, sometimes, cilia. They were rich in tonofilaments located around the nucleus or around vacuoles. Some of the cells bound large numbers of thymocytes and formed clusters identified by light microscopy as complexes type III. In contrast, epithelial cells type 3, binding 2 to 6 thymocytes only, corresponded to complexes type II under the light microscope.

Among the stromal cells of mesenchymal origin, two cell types were distinguished. The first were macrophages identified by their typical ultrastructural features and large heterophagic vacuoles (Fig. 7). The cells contained numerous lysosomes and vacuoles with electron-dense material. The other cell type showed no phagocytic properties and corresponded to interdigitating-like (IDC-like) cells. Associated with the IDC-like cells, medium-sized cells were observed; these possessed an irregular, centrally positioned nucleus and numerous finger-like protrusions of the cytoplasm (Fig. 7). Their cytoplasm contained short rough endoplasmic reticulum cisterns and numerous ribosomes. Other cells belonging to the group showed a regular outline, occasional invaginations of the cell membrane into the cytoplasm, and numerous cell organelles (Fig. 6a). The nucleus was seen at one pole of the cell. Well-developed Golgi apparatuses with accompanying vacuoles filled with electron-dense material were observed in an invagination of the nucleus. Rough endoplasmic reticulum cisterns were seen around the nucleus and at the other cell pole. Cells with intermediate characteristics were also found. Thymocytes formed contacts with IDC-like cells and macrophages via projections or adhered to the cells over a significant area. At the sites of contact, increased density of the cytoplasm could be noted (Figs. 6b, 9). Judging by the number of thymocytes bound to the surface of macrophages and IDC-like cells, they corresponded to type III complexes as observed by light microscopy.

The results of the analysis of cell complexes present in individual fractions, based on their ultrastructure, are presented in Table 2. The Table shows that complexes formed by different types of thymus stromal cells are encountered in variable proportions in individual fractions. Macrophages are most often found in the first fraction whereas IDC-like cells are mostly in the final fractions. Fractions II–VI contain similar proportions of epithelial

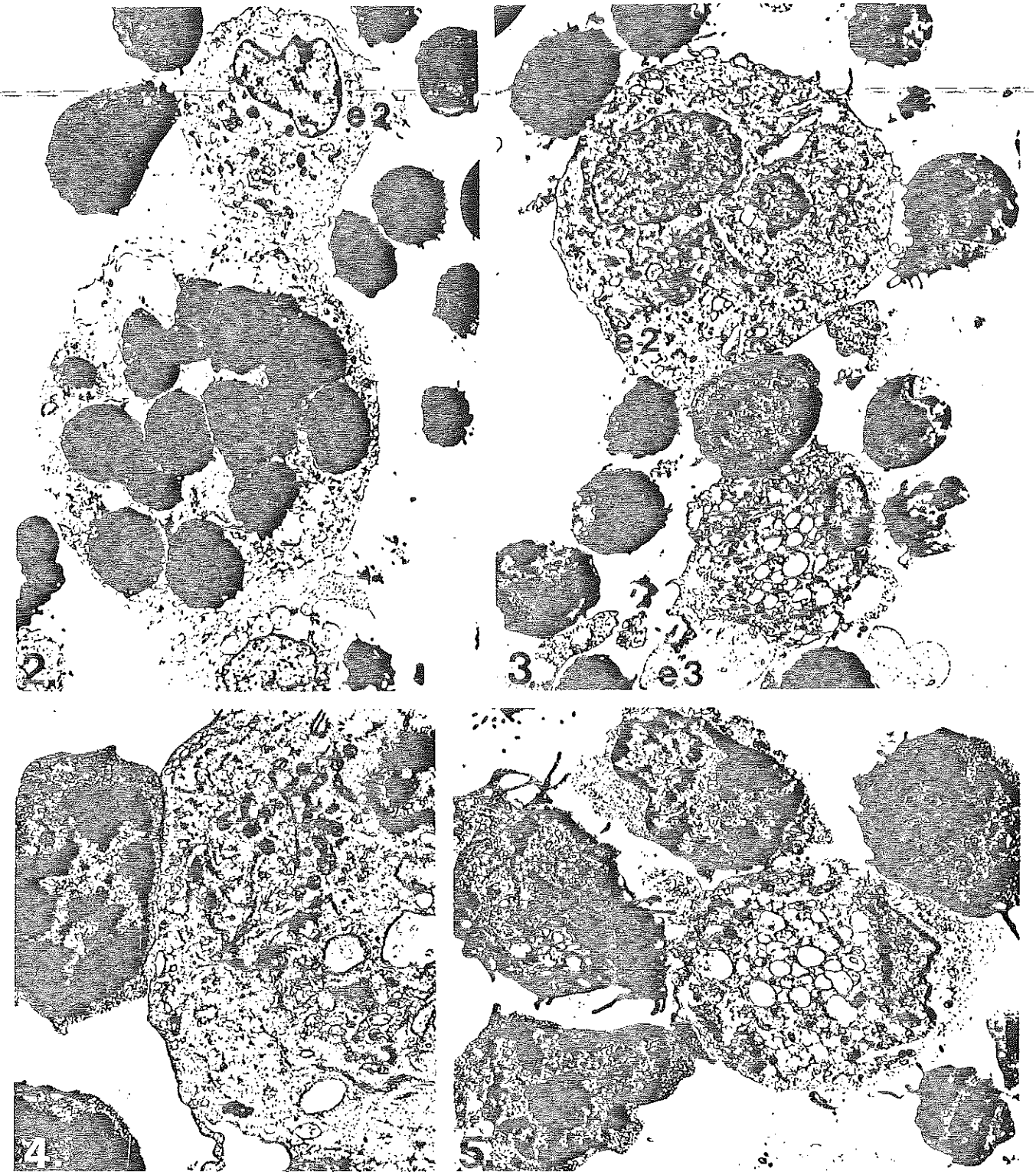
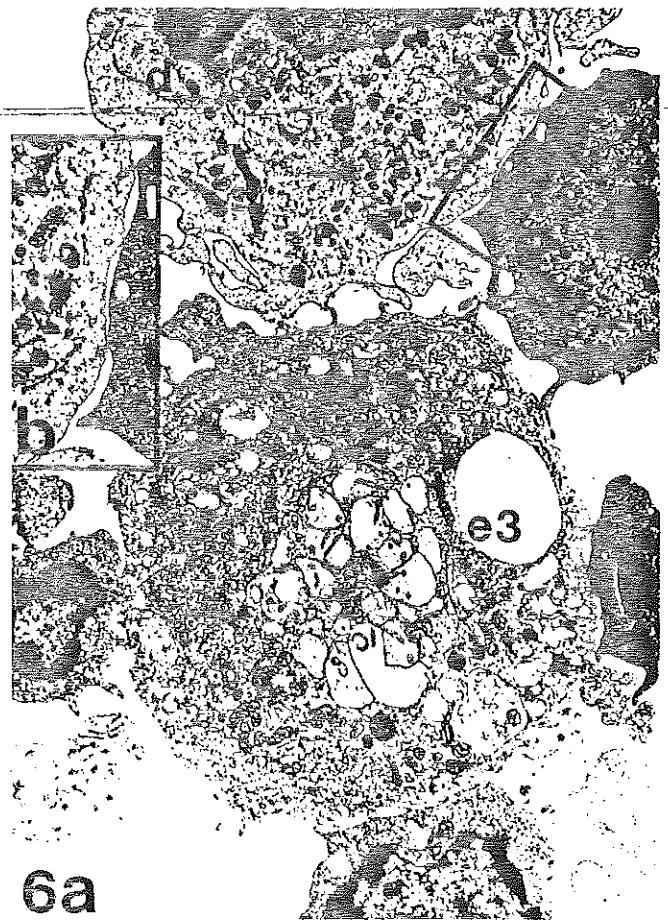


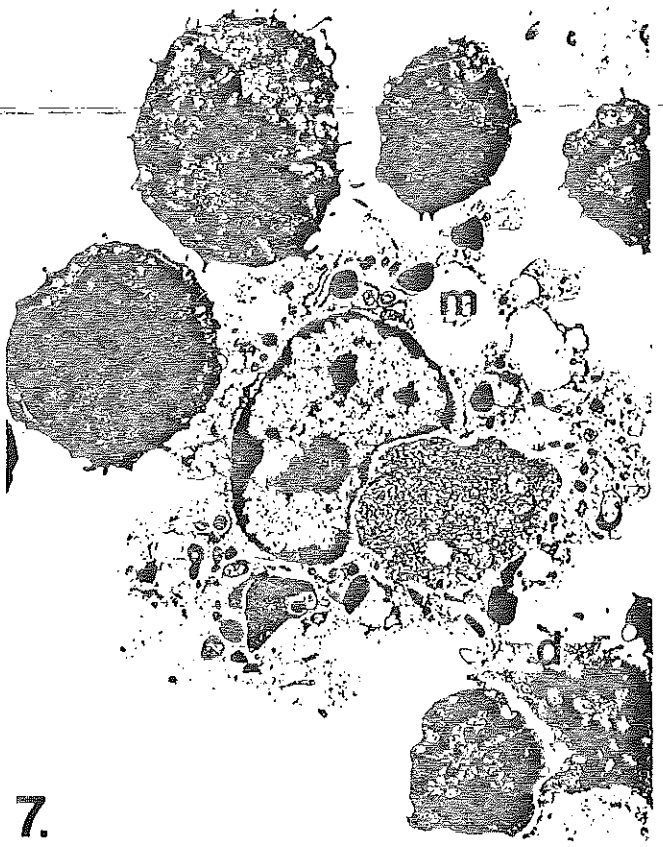
Fig. 2. Multicellular complexes of epithelial cells with thymocytes isolated from mouse thymus. In the center, an epithelial cell of type 1, at the top, an epithelial cell of type 2 (*e 2*). × 3400

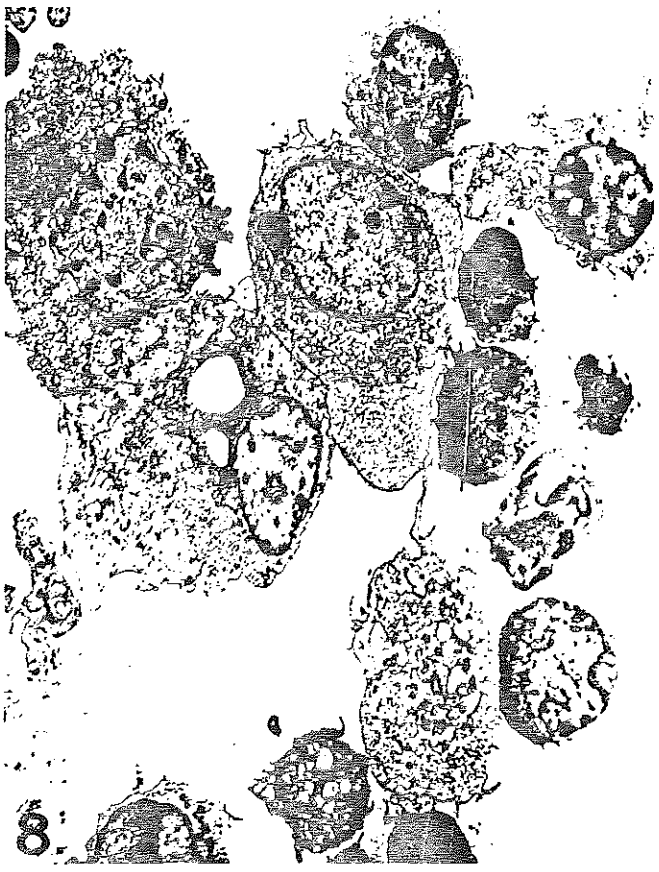
Fig. 3. Multicellular complexes of epithelial cells of type 2 (*e 2*) and type 3 (*e 3*) with thymocytes obtained from a disrupted mouse thymus. × 4400

Fig. 4. A thymocyte in direct contact with an epithelial cell of type 2. × 13500

Fig. 5. A complex of an epithelial cell of type 3 together with thymocytes isolated from mouse thymus. × 5500







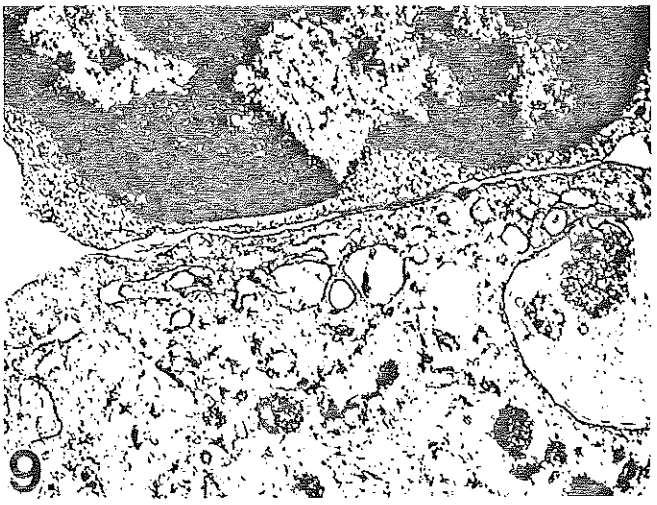


Fig. 6. A multicellular complex from mouse thymus formed by an epithelial cell of type 3 (*e 3*), an IDC-like cell (*6d*) and thymocytes. **b** Enlargement of the area indicated on **a**. a) ×6300; b) ×11100

Fig. 7. A complex of a macrophage (*m*) and thymocytes isolated from mouse thymus. On the right, an IDC-like cell (*d*) binding thymocytes by long cellular projections. ×5300

Fig. 8. Cell clumps containing several epithelial cells of type 3 together with thymocytes. ×4400

Fig. 9. A thymocyte in direct contact with the surface of a macrophage. ×22000

Table 2. Proportion of identified types of stromal cells binding thymocytes in subsequent fractions (identified in semithin sections)

Fractions	Number of analyzed complexes	Epithelial cell-thymocyte complexes			Mesenchymal cell-thymocyte complexes		Unidenti. cells
		type 1	type 2	type 3	IDC-like cells	macrophages	
		%	%	%	%	%	%
I	122	0.8	0.8	10.7	20.5	65.6	2.5
II	119	8.4	3.4	29.4	23.5	35.3	–
III	374	20.6	12.8	29.7	20.9	13.1	2.7
IV	240	15.8	19.2	27.1	32.9	3.3	2.1
V	165	7.9	35.1	27.3	25.5	4.2	–
VI	177	1.7	13.0	31.6	52.0	1.7	–

cells type 3 whereas epithelial cells type 1 are most frequently seen in fraction III and those of type 2 in fraction V.

In all fractions, cell clumps containing several central cells were encountered. Such complexes were most frequent in fractions III to V. Complexes of this type were formed by IDC-like cells binding to each other or binding epithelial cells type 3 (Fig. 6a). The fractions also contained cell clumps consisting of a few epithelial cells type 1 (TNC; some of them associated with macrophages) and epithelial cells of other types. Moreover, the latter epithelial cell types formed cell accumulations resembling Hassall's bodies. These cell accumulations bound individual thymocytes and IDC-like cells (Fig. 8).

Discussion

The procedure employed here can be used to obtain multicellular complexes composed of stromal cells and thymocytes from mouse thymuses. The cell complexes are released from the thymus during the initial incubation of the tissue in PBS. However, further isolation of the complexes requires the application of lytic enzymes. The largest number of complexes are obtained using 2 mg collagenase/1 ml PBS. At other concentrations of the enzyme or when a combination of collagenase, protease and deoxyribonuclease is used, a smaller number of complexes are obtained. Five runs, each lasting 30 min, incubating 20 mouse thymuses with collagenase at optimum concentration, at 0° C, result in complete dissociation of the organ structure and release of thymocyte-stromal cell complexes. On average, 0.4×10^5 complexes have been obtained from each thymus. Repeated experiments have yielded reproducible estimates of the total complex numbers. A preliminary analysis of such cell suspensions under the light microscope has shown that three distinct types of cell complexes can be distinguished and differences have been noted in absolute numbers and in the proportions of complexes between the fractions. This isolation procedure cannot be used to separate completely the individual types of complexes. However, individual fractions have been enriched in, or depleted of, individual types of complexes to a significant degree. For example, Fraction I is enriched in macrophages and IDC-like cells, Fractions IV and V contain mostly stromal epithelial cells, and Fraction VI only 1.7% macrophages. The sequential release of distinct complexes into individual fractions seems to reflect differences in the complex structure of the cortex and medulla of the thymus. The rate of release of the various cell complexes from both cortex and medulla may be variable, the macrophages present within the deep cortex being released faster than TNC present in the outer part of the same region (Van der Wijngaert et al. 1983). This would explain the differences in the numbers of thymic cell complexes isolated in this study as compared with those obtained by other authors (Wekerle et al. 1980; Houben-Defresne and Boniver 1983). Kyewski et al. (1982) have described thymic multicellular complexes after first dissociating the organ with collagenase IV (0.5 mg/ml) and [illegible] with a mixture of collagenase, protease and deoxyribo[illegible]lease (at 0.5, 0.5 mg/ml and 4 µg/ml, respectively). They have obtained 13.4×14^4 complexes within sequential fractions (the complexes contained rosette-like structure, formed by macrophages and dendritic cells) and in a fraction containing TNC. The differences between Kyewski's data and the results presented here seem to reflect the different temperatures used for organ dissociation and the different concentrations of the applied enzymes. It is worth noting that, under the conditions applied by Kyewski et al. (1982), both the number of TNC complexes and the number of rosettes correspond to the numbers of type I and type III complexes obtained in this study i.e., the TNC complexes and macrophage/IDC-like cell complexes, respectively.

Thus, the dissociation conditions previously described by other authors may allow the release of lymphoepithelial complexes only from the cortical zone and complexes of thymocytes with mesenchymal cells from the cortico-medullary zone. Our electron microscopic analysis has shown that thymocytes bind to the surface of both macrophages and IDC-like cells, and to various types of epithelial cells of thymic stroma. The suggested categorization of epithelial cells is superficial but includes sufficient ultrastructural features to identify the complex-forming epithelial cells in situ in individual regions of the organ. Thus, the thymus cortex contains TNC and epithelial cells type 2 and type 3, rich in microfilaments with typical vacuoles, previously described in the cortico-medullary zone and in the medulla (Hoshino 1963; Van Haelst 1967; Mandel 1970; Brelińska 1981). Moreover, some of the cells may be present within bodies resembling Hassall's corpuscles (Gaudecker and Schmale 1974).

Complexes of thymocytes with macrophages and IDC-like cells are numerous. IDC-like cells, corresponding in morphology to dendritic cells and Langerhans cells, form complexes and in situ they are present in the cortico-medullary zone and in the medulla (Kaiserling et al. 1974; Duijvestin and Hoefsmit 1981; Warchol and Brelińska 1982).

The characteristic composition of the cell complexes and the way in which central cells bind thymocytes suggest the

non-random formation of the complexes. Moreover, complex formation may reflect interrelations that exist in vivo in the mouse thymus between stromal cells and thymocytes. The presence of multicellular clusters confirms our earlier observations concerning the non-random localization of cell types within the rat thymus (Brelińska et al. 1985). Various types of stromal epithelial and mesenchymal cells form non-random complexes with each other and with thymocytes. The cell composition of such complexes is typical and corresponds to the composition of complexes released after the dissociation of the thymic structure. The physiological significance of multicellular clusters of stromal cells and thymocytes and non-random associations of defined cell types in situ remains unclear. However, the results agree with suggestions of other authors that direct contact with the various types of thymus stromal cells supports the differentiation of the contacting thymocytes within the organ (Lopez et al. 1977; Kyewski and Kaplan 1982; Van Ewijk 1984; Fink et al. 1984).

Acknowledgements. The authors wish to thank Drs. J.B. Warchol and J. Jaroszewski for valuable criticism. We are also indebted to Mrs. J. Niessen-Renson for skilful technical assistance.

References

Brelińska R (1981) Morphological types of non-lymphoid cells in the rat thymus. Folia Histochem Cytochem 19:46–47

Brelińska R, Kaczmarek E, Warchol JB, Jaroszewski J (1985) Distribution of different cell types within the rat thymus in the neonatal period of life. Cell Tissue Res 240:473–478

Duijvestijn AM, Hoefsmit ECM (1981) The micro-environment of T lymphocyte maturation. Cell Tissue Res 218:279–292

Ewijk W van (1984) Immunohistology of lymphoid and non-lymphoid cells in the thymus in relation to T lymphocyte differentiation. Am J Anat 170:311–330

Farr AG, Nakane PK (1983) Cells bearing Ia antigens in the murine thymus. An ultrastructural study. Am J Pathol 111:88–97

Fink PJ, Weissman IL, Kaplan HS, Kyewski BA (1984) The immunocompetence of murine stromal cell-associated thymocytes. J Immunol 132:2266–2272

Gaudecker B von, Schmale E-M (1974) Similarities between Hassall's corpuscles of the human thymus and the epidermis. An investigation by electron microscopy and histochemistry. Cell Tissue Res 151:347–368

Haelst UJG van (1967) Light and electron microscopic study of the normal and pathologic thymus of the rat. I. The normal thymus. Z Zellforsch 77:534–553

Hoshino T (1963) Electron microscopic studies on the epithelial reticular cells of the mouse thymus. Z Zellforsch 59:513–529

Houben-Defresne M-P, Boniver J (1983) Thymic nurse cells account for thymus dependency of preleukemic cells in mice after inoculation of radiation leukemia virus. Leuk Res 7:575–579

Kaiserling E, Stein H, Müller-Hermelink HK (1974) Interdigitating reticulum cells in the human thymus. Cell Tissue Res 155:47–55

Kyewski BA, Kaplan HS (1982) Lymphoepithelial interactions in the mouse thymus: phenotypic and kinetic studies on thymic nurse cells. J Immunol 128:2287–2294

Kyewski BA, Rouse RV, Kaplan HS (1982) Thymocyte rosettes: multicellular complexes of lymphocytes and bone marrow-derived stromal cells in the mouse thymus. Proc Natl Acad Sci USA 79:5646–5650

Lipsky PE, Rosenthal AS (1973) Macrophage-lymphocyte interaction. I. Characteristics of the antigen independent-binding of guinea pig thymocytes and lymphocytes to syngeneic macrophages. J Exp Med 138:900–924

Loor F (1979) Mouse thymus reticulo-epithelial cells in vitro: isolation, cultivation and preliminary characterization. Immunology 37:157–177

Lopez LR, Vatter AE, Talmage DW (1977) The requirement of viable thymocytes for species-specific attachment to and release from macrophages. J Immunol 119:1668–1673

Mandel T (1970) Differentiation of epithelial cells in the mouse thymus. Z Zellforsch 106:498–515

Warchol JB, Brelińska R (1982) Studies on Langerhans' cells in the rat thymus. Verh Anat Ges 76:325–326

Wekerle H, Katelsen UP (1980) Thymic nurse cells-Ia-bearing epithelium involved in T-lymphocyte differentiation? Nature 283:402–404

Wekerle H, Katelsen UP, Ernst M (1980) Thymic nurse cells. Lymphoepithelial complexes in murine thymuses: morphological and serological characterization. J Exp Med 151:925–944

Wijngaert FP, Rademakers LHPM, Schuurman HJ, Weger RA de, Kater L (1983) Identification and in situ localization of the "thymic nurse cell" in man. J Immunol 130:2348–2351

Zinkernagel RM, Callahan GN, Althage A, Cooper S, Klein PA, Klein J (1978) On the thymus in the differentiation of "H-2-self recognition" by T cells. Evidence for dual recognition? J Exp Med 147:882–896

Accepted December 18, 1985