THYMIC NURSE CELLS

Lymphoepithelial Cell Complexes in Murine Thymuses: Morphological and Serological Characterization*

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T lymphocyte differentiation is a complex developmental process involving the interaction of lymphoid cells with thymic stromal elements. Humoral factors are known to effect certain steps in intrathymic T cell differentiation (1). There is, however, little doubt that other developmental steps require direct cell contacts between differentiating thymocytes and thymic stroma cells. This is particularly true for selection processes, which are presumably involved in the generation of T lymphocyte specificity and diversity (2–5).

The mechanism of intrathymic T lymphocyte differentiation is mostly unknown. We do not even know, where in the thymus these differentiation events take place. One possible site of thymocyte differentiation is a cell type, which we have recently discovered.[1] These cells are stromal elements of extraordinary size, which may contain 25 or more small- to medium-sized lymphoid thymocytes. The engulfed thymocytes are not degraded. In contrast, they appear to encounter microenvironmental conditions, which facilitate lymphocyte survival and differentiation. We have termed these specialized stroma cells "thymic nurse cells," (TNC).[2]

In this contribution, we present ultrastructural data, which identify TNC as epithelial cells. TNC contain lymphocytes, which appear to be fully intact and metabolically active, and display high mitotic activity. The lymphocytes are completely enclosed by intact TNC membranes. It appears that specialized contact areas exist that connect internalized lymphocytes with their surrounding TNC membranes.

We furthermore present the results of experiments where we applied monoclonal antibodies against products of various subregions of the H-2 system and against a number of (T) lymphocyte differentiation markers. Antibody binding was assayed by indirect immunofluorescence with parallel flow cytofluorimetry and fluorescence microscopy.

TNC express products of the K/D regions as well as of the I-A and I-E/C regions. They lack, however, lymphocytic differentiation markers, such as Thy-1, Ly-1, Ly-2,

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[1] Wekerle, H., and U.-P. Ketelsen. 1980. Thymic nurse cells. Ia-bearing epithelium involved in T-lymphocyte differentiation. *Nature (Lond.)*. **283**:402.

[2] *Abbreviations used in this paper:* EF, external fracture; EM-H, Hepes-buffered Eagle's medium; FCS, fetal calf serum; FITC-RAMIG, fluorescein isothiocyanate-coupled rabbit anti-mouse immunoglobulin antibody(ies); MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PBS-Az, PBS with 0.2% sodium azide; PF, protoplasmic fracture; PNA, peanut agglutinin; TNC, thymic nurse cell(s); TRITC, tetramethyl-rhodamine-isothiocyanate.

Qat-4 and Qat-5, and receptors for peanut agglutinin, as well as surface immunoglobulin.

Materials and Methods

Animals. Young adult C3H/f, and C57BL/10 mice and (C3H/f × C57BL/6)F_1 hybrids were derived from the breeding facilities of the Max-Planck-Institut für Immunbiologie (Freiburg, Federal Republic of Germany). AKR/J strain mice were obtained from the Tierzuchtanstalt, Hannover, Federal Republic of Germany.

TNC Preparation

THYMUS DISSOCIATION. The mice were killed by dislocation of the neck after short ether anesthesia and the thymuses were placed in ice-cold phosphate-buffered saline (PBS). Pericapsular connective tissue, which contained blood clots, was carefully removed. The cleaned organs were minced with scissors until no gross organ fragments were discernible. The homogenate was transferred into a trypsinization bottle and agitated in 50 ml of cold PBS for 5 min. The supernate that contained the majority of mechanically dissociable thymocytes was discarded, and the sedimented fraction was resuspended in 50 ml of prewarmed trypsin solution (Dulbecco's trypsin solution: 0.25% trypsin [Boehringer Mannheim Inc., Mannheim, Federal Republic of Germany] in Ca^{++}- and Mg^{++}-free PBS) with 2 ml DNAse solution (Boehringer Mannheim Inc.). The thymus fragments were agitated for 10 min to soften the tissue and to further remove loosely bound thymocytes. Reticulum dissociation was achieved during subsequent three to four main trypsinization rounds that took place in 50 ml warm trypsin/DNAse solution within 20 min of each other (6). After each round, the supernates that contained dissociated cells were collected, and the sediments were subjected to further trypsinization until complete disintegration of the organs. The cell fractions that contained sizeable amounts of TNC and other nonlymphoid reticulum cells within a majority of thymocytes were pooled and stored in cold Hepes-buffered Eagle's medium (EM-H).

ISOLATION OF TNC. Because TNC are, by many times, larger cells than thymocytes, macrophages, or conventional thymic reticulum cells, they could be easily isolated by a simple four-step 1-g sedimentation procedure. TNC-containing trypsin fractions (usually 1–1.5 × 10^9 total cells) were pooled and concentrated in a vol of 3 ml EM-H. This concentrated suspension was gently layered over 10 ml of undiluted, heat-inactivated fetal calf serum (FCS, LS Service, Munich, Federal Republic of Germany) in conical 40-ml glass centrifuge tubes. The sediment that contained TNC was collected after a 15-min incubation at room temperature, resuspended in 0.7 ml EM-H, overlayered over a column of 5 ml FCS in a 12-ml Falcon plastic tube (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.), and was allowed to sediment for 10 min. This sedimentation procedure was repeated twice and resulted in a population that contained >80% TNC (Table I).

ELECTRON MICROSCOPY. TNC populations were fixed in 3% glutaraldehyde (veronal acetate buffer, pH 7.3, 4°C, 1 h). Postfixation was done in 1% OsO_4. After dehydration in an alcohol row, the cells were embedded in Epon (Shell Chemical Co., Houston, Tex.). Thin sections were stained in methylen blue-azur II. Ultrathin sections were stained with uranyl acetate and lead citrate.

For freeze fracturing, glutaraldehyde-fixed cells were glycerinated (30% glycerol, 90 min) and frozen directly in liquid H Freon 22 (E. I. du Pont de Nemours, Inc., Wilmington, Del.) on copper dishes. Freeze fracturing and replication was done at −100°C specimen temperature with a Balzers freeze-etch apparatus (Balzers Aktiengesellschaft für Hochvakuumtechnik und Dünne Schichten, Balzers, Liechtenstein). Shadowing was performed with platinum carbon at an angle of ~45°C. The replicas were examined in a Philips EM 400 (Philips Eindhoven, The Netherlands).

Ferritin Labeling of TNC Membranes. TNC suspended in EM-H (4 × 10^5 cells in 1 ml) were mildly fixed by adding 0.3 ml 3% glutaraldehyde (15 min, 4°C). The cells were washed in veronal buffer and resuspended in 1 ml of this buffer that contained 5 mg of cationized ferritin (Miles-Yeda, Rehovoth, Israel) for 30 min at room temperature (7). The cells were washed twice and processed for electron microscopy as indicated above.

Antisera. Monoclonal antisera against Thy-1.2 were obtained from Olac, Ltd. (Bicester,

TABLE I

Schematic Procedure of TNC Isolation by Repeated 1-g Sedimentation

Step	FCS	Suspension	Sedimentation	TNC	Total cells
	ml	*ml*	*min*	%	
1	10	3	15	0.3	170×10^6
2	5	0.7	10	3	15×10^6
3	5	0.5	10	30	1.5×10^6
4	2	0.2	10	80	0.7×10^6

Oxon, England). The monoclonals against major histocompatibility complex (MHC) and lymphocyte differentiation determinants were kindly provided by Dr. G. J. Hämmerling (Institut für Genetik, Köln, Federal Republic of Germany). They have been described in detail (8, 9). Their main features are listed in Table II. A fluorescein isothiocyanate-coupled rabbit anti-mouse immunoglobulin antibody preparation (FITC-RAMIG) was purchased from Miles-Yeda.

Indirect Immunofluorescence Labeling. Semipurified TNC populations that contained 30% TNC along with macrophages, smaller lymphoid, and reticular elements were washed and resuspended in PBS with 0.2% sodium azide (PBS-Az). Equal vol of 200 μl, containing ~50×10^3 TNC each, were transferred into Eppendorf micro test tubes (Metheler und Hinz, Hamburg, Federal Republic of Germany). We added 10 μl of undiluted hybridoma ascites fluid, which contained the monoclonal antibodies. After 30 min of incubation at ice temperature, the antibody-treated cells were washed three times in 1 ml of PBS-Az, resuspended in 100 μl of PBS-Az, and treated with 10 μl of FITC-RAMIG dilution (diluted 1:6 in PBS-Az). After another period of 30 min on ice, the cells were washed again three times in 1 ml of PBS-Az and resuspended in 50 μl of PBS-Az. One-half of the suspension was introduced into flat cover-slip chambers for fluorescence microscopy, and the other half was used for flow cytofluorimetry.

FITC-Peanut Agglutinin (PNA) Labeling of TNC. FITC-conjugated PNA was provided by Sigma Chemical Co., St. Louis, Mo. We added 10 μl of FITC-PNA solution to 200 μl TNC suspension (~50×10^3 TNC in PBS-Az). After a 30-min incubation at ice temperature, the cells were washed thrice, and then screened under the fluorescence microscope.

Fluorescence Microscopy. The labeled TNC preparates were viewed in a Zeiss epifluorescence microscope (Carl Zeiss, Inc., New York). The cells were first photographed in visible light, and subsequently in exciting ultraviolet light. We used Kodak 3X films (Eastman Kodak Co., Rochester, N. Y.), with exposure of fluorescent cells of 60 s. We scored fluorescence intensities (Table II) as follows: ++ (bright, distinct ring-shaped fluorescence); + (fluorescent rings still discernible); ± (vague, diffuse fluorescence, without ring formation); − (no fluorescence at all).

Flow Cytofluorimetry. Light scattering and fluorescence intensity of TNC were simultaneously determined with a cytofluorograph (model 4800 A, Ortho Instruments, Westwood, Mass.). Fluorescence was excited by an argon laser beam (50 mW output at 488 nm). The TNC preparations were diluted in a total of 5 ml of PBS, and measured in portions of 1.5 ml each. Distribution diagrams (dot plots) were photographed as soon as a total cell content of 50,000 was reached. Light scattering (reflecting cell size) is given on the abscissa, and fluorescence intensity is given on the ordinate.

Tetramethyl-Rhodamine-Isothiocyanate (TRITC)-labeling of Zymosan. We suspended 20 mg of zymosan (Lot DZ-3185, Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) in 30 ml of boiling 0.5 M sodium bicarbonate solution (pH 9.5). Total deaggregation of the zymosan particles was obtained by short ultrasonication. After cooling the suspension, we added 1.6 mg of TRITC (Nordic Laboratories, Tilburg, Netherlands). The mixture was stirred overnight at 4°C. The zymosan particles were centrifuged (2,000 rpm, 10 min), and washed five times in ice-cold acetone water (three parts distilled water, one part acetone), before being resuspended in 1 ml of 0.5 M sodium bicarbonate solution. This stock suspension was stored at 4°C.

Phagocytosis Assay. We resuspended the cells (1×10^6) in 1 ml of EM-H enriched with 10%

TABLE II

Surface Antigens on TNC, Thymic Macrophages, and Lymphoid Thymocytes

			Mice											
			(C3H/f × C57BL/6)F_1			C3H/f			C57BL/6			AKR/J		
			TNC	Macrophages	Thymocytes	TNC	Macrophages	Thymocytes	TNC	Macrophages	Thymocytes	TNC	Macrophages	Thymocytes
Membrane Ig	—	FITC-RAMIG	−	−	−	−	−	−	−	−	−	−	−	−
Thy-1.2	F7D5	FITC-RAMIG	−	−	++	−	−	++	−	−	++	−	−	−
H-2K^k(H-2.25)	H100-27.R9	FITC-RAMIG	++	++	±	++	++	±	−	−	−	++	++	±
H-2K/D^k(H-2.5)	H100-30.R3	FITC-RAMIG	++	++	±	++	++	±	−	−	−	++	++	±
I-A^k(Ia.19)	H116-32	FITC-RAMIG	++	++	−	++	++	−	±	−	−	++	++	−
I-A^k(Ia.2)	H118-49	FITC-RAMIG	++	++	−	++	++	−	−	−	−	++	++	−
I-E/C^k(Ia.7)	13/4	FITC-RAMIG	+	+	−	+	+	−	−	−	−	+	+	−
I-A^b(Ia.9)	B17-123.R4	FITC-RAMIG	++	++	−	−	−	−	++	++	−	−	−	−
I-A^b(Ia.8)	B17-263.R6	FITC-RAMIG	++	++	−	−	−	−	++	++	−	−	−	−
H-2D^b(H-2.2)	B22-249.R1	FITC-RAMIG	++	++	±	−	−	−	++	++	±	−	−	−
Qat-4	B16-146.R1	FITC-RAMIG	−	−	−	−	−	−	−	−	−	−	−	−
Qat-5	B16-167.R10	FITC-RAMIG	−	−	−	−	−	−	−	−	−	−	−	−
Ly-1.1	W/3$^-$	FITC-RAMIG	−	−	±	−	−	±	−	−	−	−	−	−
Ly-2.2	19/178	FITC-RAMIG	−	−	+	−	−	−	−	−	+	−	−	+
PNA	—	—	−	−	+	−	−	+	−	−	+	−	−	+

cytofluorometry. Scoring of immunofluorescence, as indicated in Materials and Methods.

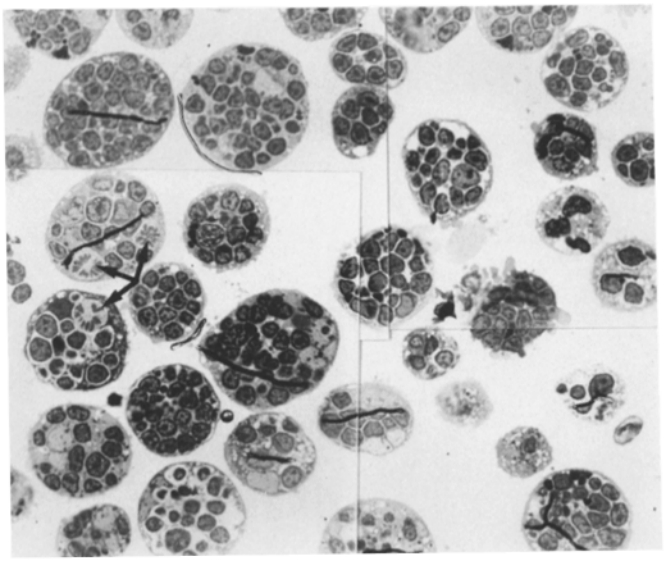

FIG. 1. Purified TNC population that contains predominantly intact lymphoid cells, including three mitotic figures (arrows). Semithin section of Epon-embedded single-cell suspension. Staining: methylen blue-azur II.

FCS, and added 20 μl TRITC-zymosan stock suspension. After a 30-min incubation at 37°C in normal atmosphere, the cells were centrifuged, resuspended in 0.5 ml EM-H, and underlayered with 2 ml of FCS. Within 15 min, most of the living cells sedimented to the bottom of the tubes, whereas free zymosan particles remained in the upper layer. As a result of their content of bright red fluorescent particles, phagocytic cells were identified under the fluorescence microscope.

Results

Morphology of TNC (Light Microscopy). A semithin section through an enriched preparation of TNC is shown in Fig. 1. This population is composed of TNC that vary considerably in size and in regard to the number of lymphocytes engulfed. Whereas some small TNC contain only one lymphocyte in this section plane, large cells can show up to 25 sectioned lymphocytes. In most cases, one TNC nucleus is visible. TNC may possess, however, two nuclei, as suggested by these sections.

Ultrastructure of TNC. TNC resemble by many morphological criteria epithelial cells from the thymic cortex (10, 11). Their cytoplasm stains relatively light, and shows characteristic vacuoles as well as dense bodies. Many vacuoles contain a granulated osmiophilic material. Strings of tonofilaments are observed particularly near the outside membrane, and, much less frequently, in the intercaveolar cytoplasmic parts. In may cases, the nuclei contain nucleoli arranged in a reticular pattern (Figs. 2 and 3). In freeze-etch preparations, the outer membranes of TNC show particle arrays, which resemble tight junctions described in various epithelial tissues (Fig. 8 a). From their appearance they could represent the remnants of trypsin-cleaved intercellular connections (12).

The Engulfed Lymphocytes. The majority of the engulfed lymphocytes appears morphologically as intact, small to medium lymphocytes. The scanty cytoplasm frequently contains disseminated ribosomal clusters. The mitochondria are intact, not

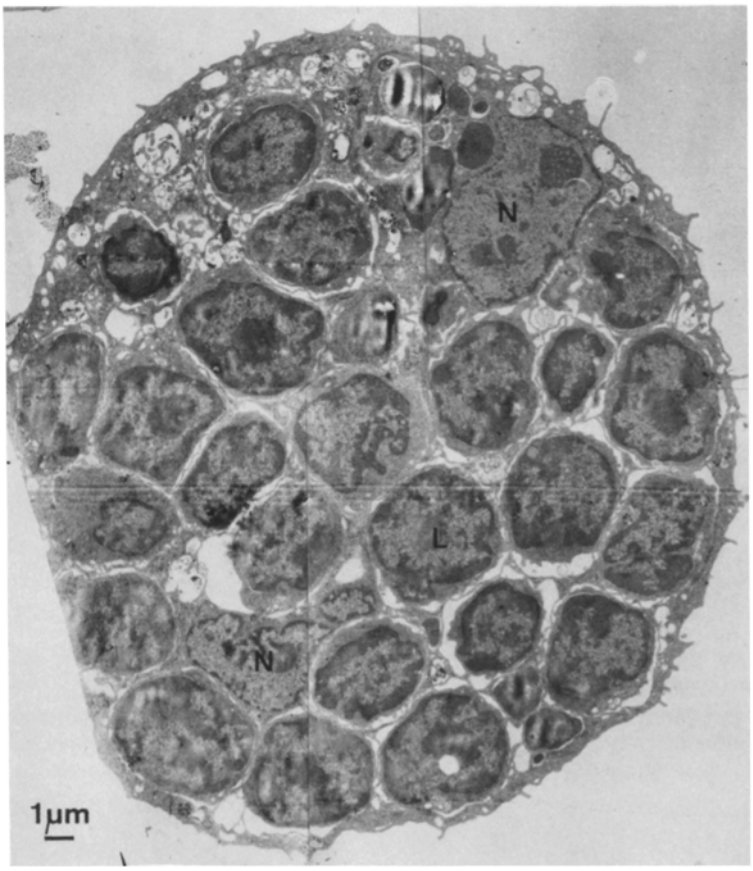

FIG. 2. Single, large TNC presumably sectioned through central plane. Two TNC nuclei (N) displaying typical reticular nucleolus and peripherally distributed chromatin. Cytoplasmic specializations include vesicles filled with coarse material, and tonofilament bundles (for details see Figs. 3, 4, 5, and 6). L, lymphocyte. Staining: uranyl acetate and lead citrate.

swollen. Mitoses can be noted frequently (Figs. 1 and 3). We have never found higher differentiated lymphoid cells, such as plasma cells or nonlymphoid cells.

Complete Seclusion of Internalized Lymphocytes. To test, whether the surrounding of the lymphoid cells by TNC cytoplasm is indeed complete, or, whether communications of the caveolar spaces with the extracellular environment exist, TNC were incubated in culture medium that contained 5 mg/ml cationized ferritin. Cationized ferritin is known to diffuse into spaces that communicate with the extracellular environment, and, because of their negative charge, finally is adsorbed to the cell membrane surfaces that line these spaces (7). Fig. 4 shows an ultrathin section of a TNC preparation, which has been treated with cationized ferritin. The outside membrane of the TNC is homogeneously lined with electron-dense ferritin molecules. In contrast, the closely adjacent membranes of a TNC caveola that surrounds a small lymphocyte are free of ferritin. Thus, the engulfed lymphocytes are completely isolated from the outside space.

Membrane Interactions between TNC and Internalized Lymphocytes. In ultrathin sections two distinct types of contact between lymphocyte and surrounding TNC caveolar

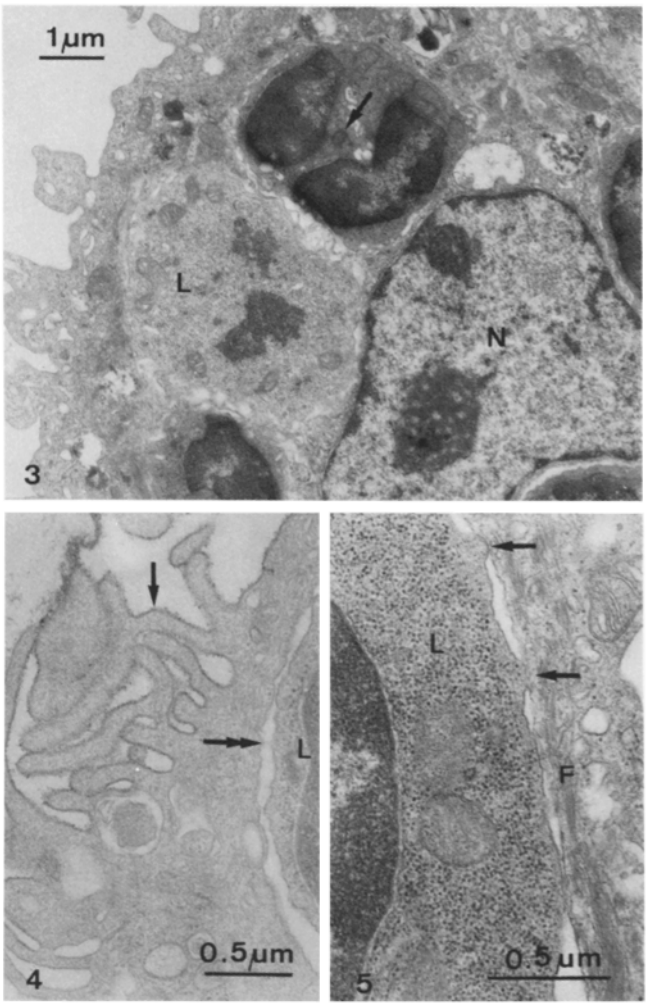

FIG. 3. Mitotic proliferation activity of internalized lymphoid cells. One mitotic figure (L) and one lymphocyte containing centriole (arrow). Characteristics of TNC nucleus (N) as in Fig. 2. Cytoplasmic vesicles. Staining: uranyl acetate and lead citrate.

FIG. 4. Complete seclusion of internalized lymphocyte (L). Labeling of TNC outside plasma membrane with cationized ferritin (arrow). No ferritin molecules have permeated into the perilymphocytic TNC caveolar space (double arrow). Staining: uranyl acetate, solely.

FIG. 5. Circumscript contacts between internalized lymphocyte (L) and surrounding TNC caveolar membrane (arrows). Note tonofilament bundles (F) in the TNC cytoplasm. Staining: uranyl acetate, and lead citrate.

membrane could be discerned. Some lymphocytes, mainly of medium size, are completely surrounded by a tightly fitting caveolar membrane (Fig. 6). Two unit membranes can be distinguished all along the contact zone, without any signs of membrane fusion, or discontinuation. In most cases however, contact between lymphocytes and the TNC caveolar membrane is restricted to multiple, circumscript areas. At high magnifications, the membranes within these contact areas appear to be condensated, but lack definite substructures (Fig. 5).

Freeze-fracture studies provided further insight into the TNC caveolar membrane.

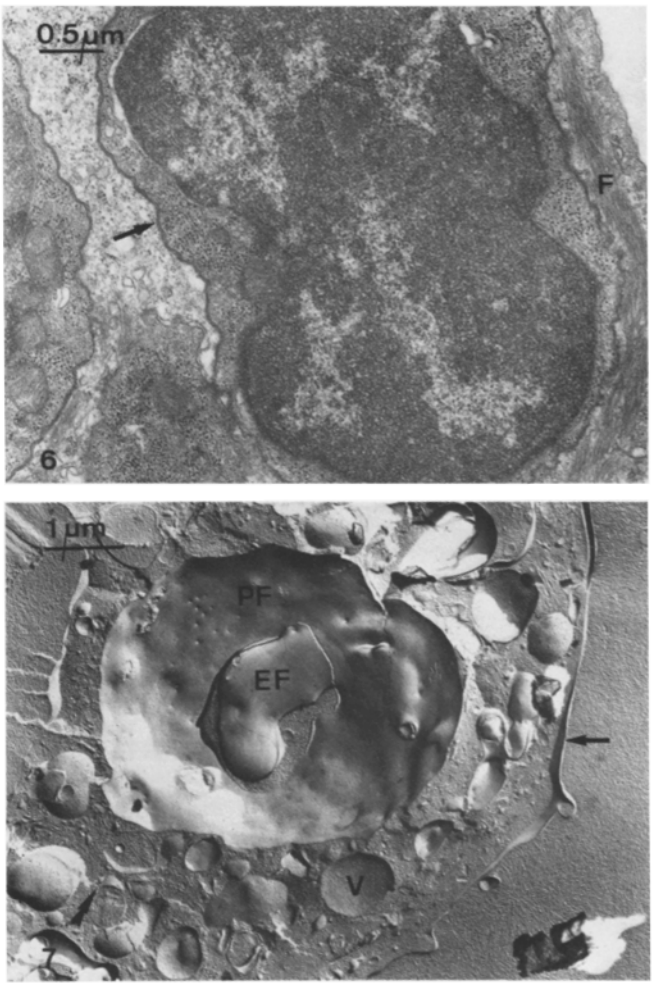

FIG. 6. Continuous contact between internalized lymphocyte and surrounding TNC caveolar membrane (arrow). Both adjacent membranes run completely parallel but do not show signs of fusion or intermembraneous substructures. Cytoplasmic tonofilament bundle (F). Staining: uranyl acetate and lead citrate.

FIG. 7. TNC caveolum that contains part of internalized lymphocyte membrane. Freeze-fractured peripheral area of a TNC. PF face of TNC caveolar membrane (PF) forms concave bowl that contains in its center a residual part of the external membrane fracture face (EF) of the internalized lymphocyte. Note fractured vesicles (V) in the TNC cytoplasm and the limiting outer plasma membrane (arrow). Direction of shadowing: double arrow.

Fig. 7 shows a protoplasmic fracture (PF) face (convex fracture) of a TNC caveolar membrane, with a residual part of the external fracture (EF) face of an enclosed lymphocyte. Prominent features of the TNC caveolar membranes are invaginations and characteristic particle arrangements. Figs. 7 and 8b display typical membrane invaginations, which usually are arranged in groups. Our present findings do not allow us to decide whether the caveolar membrane invaginations represent exo- or endocytotic events.

Intramembraneous particles were distributed in several arrangements. Besides

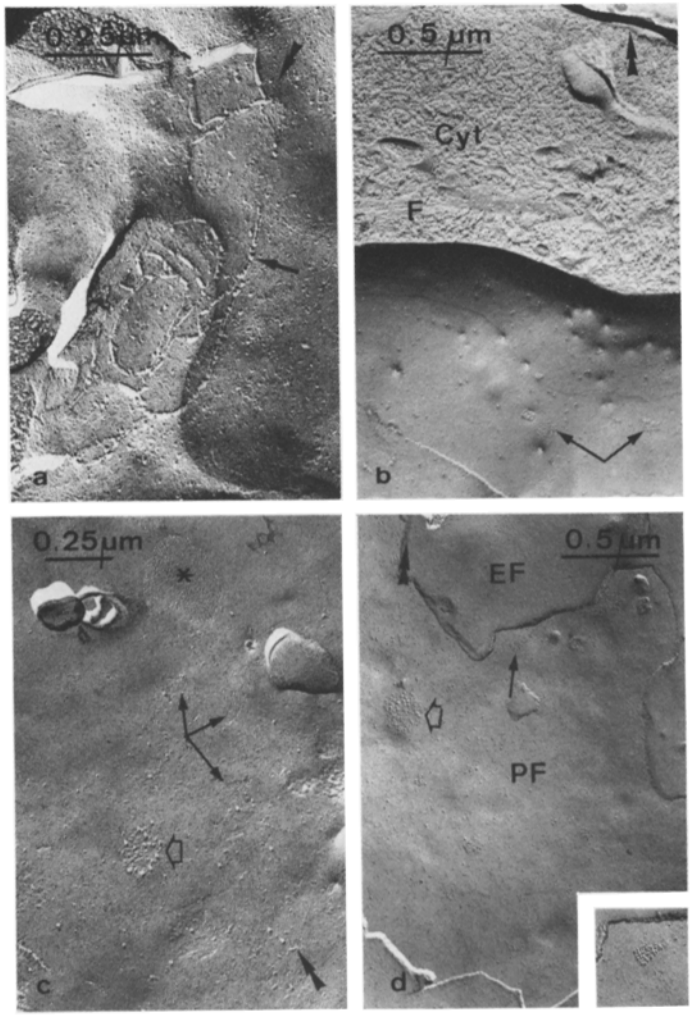

FIG. 8. Membrane specializations of outside and caveolar TNC membranes. (a) Tight-junction-like branched strands of single particles of P face of a TNC outer plasma membrane (arrow). (b) TNC caveolum with adjacent cytoplasm (Cyt). The caveolar protoplasmic face displays as typical specializations invaginations and, besides single membrane particles, particles arranged in square arrays (arrows). Note longitudinally fractured tonofilaments (F) in cytoplasm. (c) Specialization of the E face of a TNC caveolar membrane. Pit impressions of intramembraneous particles organized in square arrays (arrows). Impression of a possible gap junction (asterisk). Amorphous aggregation of heterogeneous particles (open arrow). (d) Square arrays of particles on PF face of caveolar membrane (arrow and inset) besides single particles and heterogeneous particle aggregation (open arrow). EF face belonging to the plasma membrane of the internalized lymphocyte does not display analogous specialized particle arrangements. Direction of shadowing: double arrows.

single, disseminated intramembraneous particles (70–100 Å diameter), we found patches of aggregated particles (Fig. 8c) of varying sizes. These membrane specializations closely resemble spot desmosomes of various tissues (13). It should be noted that these particle aggregates were observed both on PF and on EF faces of the TNC caveolar membrane (Fig. 8c and d). A second type of intramembraneous particle

aggregates is shown in Fig. 8c. The EF face of a TNC caveolar membrane contains an oval arrangement of closely packed pits of uniformly sized particles, which resemble gap junctional structures. A final, quite unusual membrane specialization that was regularly found on TNC caveolar membranes is shown on Fig. 8b, c, and d. On PF faces, uniform particles organized in square arrays are seen. They correspond to similar arrays of pits on EF faces, which, thus, seem to reflect the impressions of the particles shown on PF faces.

Membrane Antigen Determinations. We determined possible thymus cell determinants with monoclonal hybridoma antibodies as probes, and conventional FITC-RAMIG as label. Semiquantification was achieved in a flow cytofluorograph. To facilitate the interpretation of the cytofluorographic results, we screened the populations morphologically, applying fluorescence microscopy.

The cell populations analyzed in these investigations were semipurified TNC preparations. They contained mainly TNC, as well as similar numbers of free lymphoid thymocytes, macrophages, and reticular elements. Figs. 9A and 10A show that neither thymocytes nor TNC were fluorescent, when treated with FITC-RAMIG alone. Hence, they did not express surface immunoglobulin. Anti-Thy-1.2 antibody treatment followed by FITC-RAMIG lead to a typical distribution pattern in C57BL/6 mice, which express Thy-1.2 specificity. Fig. 10B shows a population composed of two distinct groups: one intensely fluorescent, grouped along a diagonal axis in the plot, and the other one negative. Immunofluorescence microscopy reveals that the immunofluorescent group contains the thymocytes, which are mainly grouped in cell aggregates of varying size. AKR/J cells, phenotypically Thy-1.1, were completely negative (Fig. 9B).

We determined the expression of H-2 specificities coded for by several subloci. In all cases, classical K- and D-region antigens were strongly and specifically expressed on TNC. Thus specificity H-2.25 of H-$2K^k$ was present on AKR/J cells (Fig. 9C), but lacking on C57BL/6 cells (Fig. 10C). H-2 K/D antigens were, however, only very weakly expressed on thymocytes. Expression on thymocytes was detectable on the cytofluorograph plots as a confluence of the intensely fluorescent large TNC population area with one of the weakly fluorescent, small thymocytes.

The highest fluorescence intensities were found for the I-A-region determinants and both of the H-2^k and H-2^b haplotypes. This was found with two different hybridoma antibodies directed against different specificities of two different haplotypes, H-2^k and H-2^b. In the dot plots, the bright TNC populations are distinctly separated from the negative thymocytes (Figs. 10F and G, and 9D and E). Immunofluorescence microscopy did not detect any staining of thymocytes. The one anti-I-E/C antibody, directed against the H-2^k haplotype, was in concordance with the anti-I-A-region antibodies, as far as specific staining of TNC and the lack of it on thymocytes, was concerned. The staining intensity was, however, much weaker than the one of the I-A region antibodies (Fig. 9H).

Among the nonlymphoid thymic cells, macrophages are a substantial population. Because the antigen formula of TNC was identical with the one reported for macrophages from the peripheral immune system (14, 15), we found it necessary to directly compare TNC and thymic macrophages with a fluorescence double-label test. Phagocytic cells were visualized by their ability to ingest rhodamine-labeled zymosan,

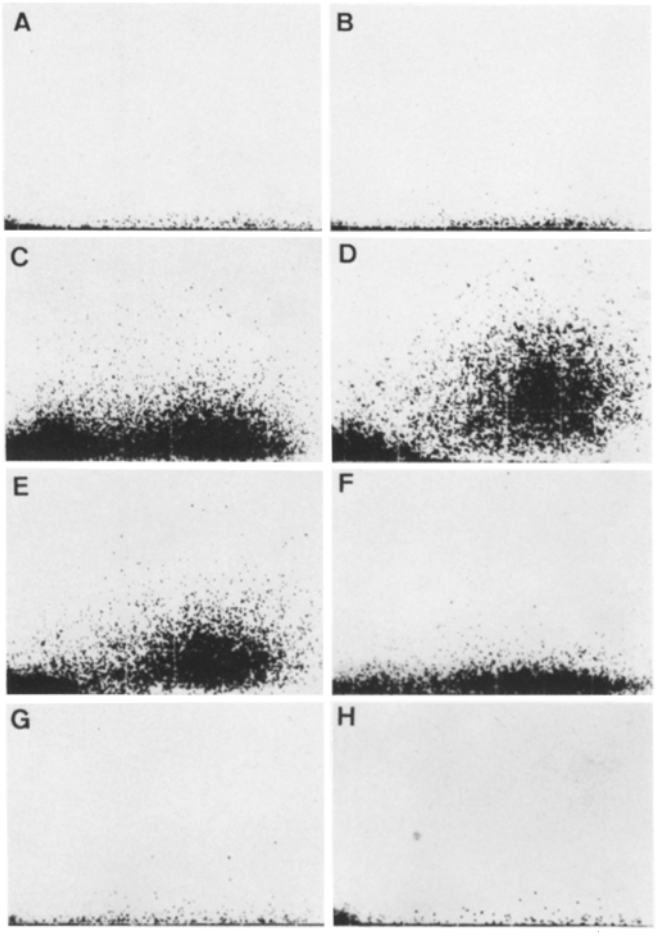

FIG. 9. Cytofluorometric determination of surface antigens on AKR/J TNC. Light scattering of single cells is given on the abscissas, and fluorescence intensity on the ordinates. The cells were treated with alone (A) FITC-RAMIG, or, before FITC-RAMIG treatment, with (B) anti-Thy-1.2, (C) anti-H-2K^k, (D) anti-I-A^k (Ia. 19), (E) anti-I-A^k (Ia. 2), (F) anti-I-E/C (Ia. 7), (G) anti-I-A^b (Ia. 9), and (H) anti-H-2D^b (H-2.2).

and the surface antigens were demonstrated, as in our previous experiments, by applying monoclonal hybridoma antibodies and FITC-RAMIG.

Our analyses indeed confirm the serologic similarity between TNC and macrophages. Both cell types express all H-2-subregion determinants detectable by our antibodies. Both populations lack lymphocyte-specific antigens, such as Thy-1.2, Ly-1.1, Ly-2.2, Qat-4, and Qat-5. Our experiments indicate, however, an important functional difference between TNC and macrophages. TNC were never seen to ingest zymosan, and, in contrast, phagocytic cells never contain morphologically intact autochthonous thymocytes.

Our collected data are summarized in Table II. They establish that TNC express all H-2-subregion determinants tested for. Determinant expression was highly intensive in all cases, except the weaker binding I-E/C antibodies. Binding was fully

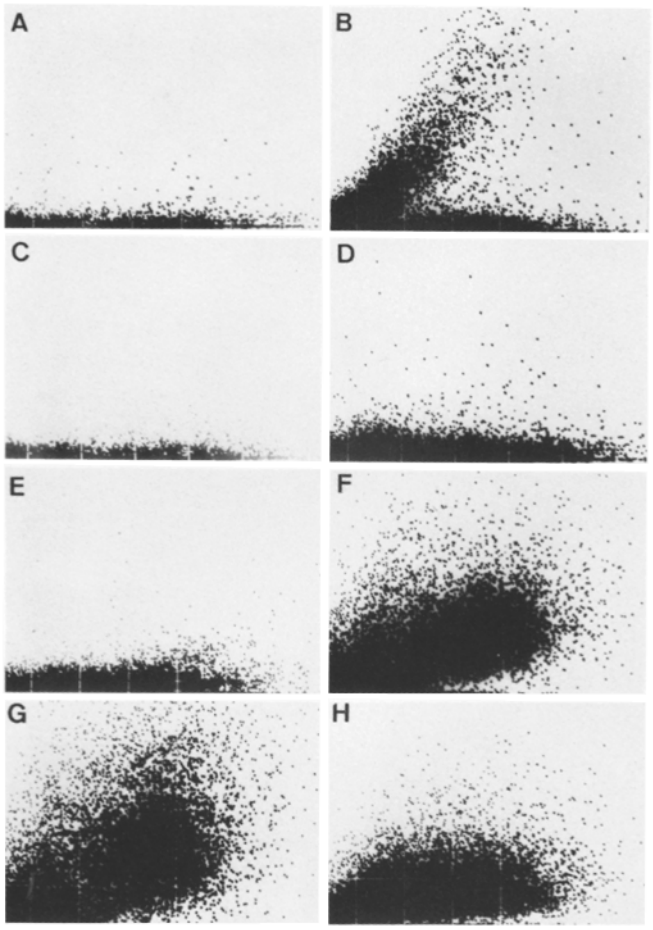

FIG. 10. H-2 determinants on C57BL/6 TNC. Cytofluorometric analysis as in Fig. 1. Pretreatment of the cells: (A) FITC-RAMIG alone, (B) anti-Thy-1.2, (C) anti-H-2K^k (H-2.25), (D) anti-I-A^k (Ia. 19), (E) anti-I-E/C (Ia. 7), (F) anti-I-A^b (Ia. 9), (G) anti-I-A^h (Ia. 8), and (H) anti-H-2D^b (H-2.2).

haplotype specific. TNC, however, do not express any of the lymphocyte differentiation markers investigated. These include Ig for B lymphocytes; as well as the T cell markers Thy-1.2, Ly-1.1, and Ly-2.2, and, finally, the newly described specificities Qat-4 and Qat-5 (16). TNC do not bind PNA, a marker for immunoincompetent murine thymocytes (17, 18).

Discussion

Thymuses from normal rats and mice contain stromal cells of large size, which have internalized high numbers of small- to medium-sized lymphoid thymocytes. These TNC are found as regular and substantial components in all normal rat and mouse thymuses. TNC can be isolated by repeated tryptic dissociation of thymus fragments and subsequent gravity sedimentation. As a rule, we recover $\sim 10^4$ intact TNC from one young adult mouse thymus. These TNC together may harbor an estimated $1-2 \times 10^5$ lymphoid thymocytes.

Incorporation of cells by other cells can be the consequence of either phagocytosis, or, alternatively, of active invasion. In regard to TNC, our observations suggest that the latter is correct. First of all, the internalized lymphocytes were, in their majority, morphologically intact, many of them undergoing mitosis. There was no evidence for a logical phagocytic degradation process in TNC. Furthermore, by morphological and functional criteria, TNC resemble epithelial cells rather than phagocytes. Ultrastructurally, well-ordered bundles of tonofilaments are prominent in their cytoplasm, in particular beneath the outside membrane. By using freeze-etch techniques, we found linear particle arrays on fracture faces of the outside membranes, which can be interpreted as remnants of trypsin-cleaved tight junctions (12). Tonofilament bundles and tight junctions are commonly found in epithelial cells, but not in macrophages. Vacuoles that contain coarse granular material of undetermined origin are typical for TNC, as they are for epithelial cells from the thymus cortex (10, 11). Mandel (19) found many dividing thymocytes in close association with cortical dendritic reticulum cells. Moreover, unlike macrophages, cultured TNC do not readily adhere to untreated, or collagen-coated surfaces. TNC first tend to form intercellular aggregates, and spread on culture-plate bottoms only subsequently. Finally, in long-term cultures of highly pure TNC fractions, epithelium monolayers are formed, which lack mesenchymal elements, and, most importantly, are devoid of macrophages. It should be pointed out, that macrophages and fibroblasts are the predominant monolayer-forming cell type in mechanically dissociated, conventional thymus monolayer cultures.

Active invasion of various cells by lymphoid cells, emperipolesis (20), has been described in a number of cases, primarily in tissue culture. In most instances, normal or neoplastic monolayer-forming cells were in close contact with normal or neoplastic lymphoid cells (21). The coating of target cells with antibodies was found to enhance emperipolesis (22, 23). In all cases of emperipolesis, it was difficult to determine, whether the associated lymphocytes were indeed completely incorporated, or, whether they merely bulged into the surface membrane, and were touched there by a tangential section (24). This possibility was ruled out in our experiments by incubation of TNC preparates in cationized ferritin solution (7). Ferritin diffuses into those spaces that communicate with the extracellular space and will line adjacent cell-membrane portions. This method, which, for example, was used to demonstrate the communication of the myotube T tubular system (25), revealed that the perilymphocytic caveolae within TNC are completely secluded from outside. Exclusion of fluorescein-labeled antibodies and lectins (anti-Thy-1.2 and PNA) from TNC caveolae complement these findings on microscopical level.

Although many techniques have been developed to dissociate and culture thymic reticulum (26–32), TNC have not been described before. This is probably because TNC are firmly anchored within the thymic stroma. They are dissociated only after repeated trypsinization rounds. Small reticular elements, in contrast, particularly of mesenchymal origin, are released much more easily. Our demonstration of membrane specializations on outer membranes of TNC, which resemble epithelial tight junctions (12), lend morphological support to the strong attachment to the stromal network.

Why have TNC been overseen *in situ*? First, and most trivially, TNC could be a preparation artifact, e.g., caused by our dissociation schedules. This is improbable for at least two reasons. We do not know of a precedent in which trypsinization can

induce invasion of stromal cells by lymphocytes. Stromal-lymphoid cell complexes were only found in thymic preparations, but never in corresponding dissociates of other lymphoid organs. TNC formation is the exclusive property of a specialized thymic stroma cell, which was further emphasized by the fact that similar cellular complexes can be formed in thymic cultures (H. Wekerle. Unpublished observations.). The most cogent argument against a trypsinization artifact is, however, our recent finding of TNC in thymus preparations, which were dissociated only mechanically, without enzymatic digestion (H. Wekerle and U.-P. Ketelsen. Unpublished observations.).

Demonstration of TNC *in situ* presumably encounters considerable technical difficulties. Normal thymuses are usually overcrowded with lymphoid thymocytes, and it is often difficult to clearly delineate reticulum cells under the light microscope. In most electron microscopic studies, the inability to screen serial sections may have led to interpretations of TNC cytoplasmic septae as "dendritic processes separating small lymphocytes" (10, 11). However, epithelial-lymphoid cell complexes strongly resembling our isolated TNC were described by Hwang et al. (33), who found these cells, interestingly, near thymic vascular elements. Thymic lymphocyte emperipolesis has indeed been described in a few isolated reports. Klug (34) found that in thymuses from irradiated adrenalectomized rats apparently intact lymphocytes are contained within a certain type of reticulum cells. Llombart-Bosch (35) and Levine et al. (36) showed thymocytes within neoplastic reticulum cells and, more recently, Seemayer et al. (38) demonstrated lymphocytic emperipolesis in thymus reticulum from mice undergoing graft-versus-host disease. In all these cases, thymuses were investigated that, for various reasons, had undergone marked lymphoid depletion.

The residence of viable lymphocytes within specialized epithelium cells reminds one of intracellular symbiosis recently discussed by Smith (38), who pointed out that any cell that sojourns within another viable cell lives in an unusual and extreme environment. All cases of intracellular symbiosis must share some essential common features. (*a*) Invasion of the host cell must be preceded by mutual recognition, usually via complementary surface receptors. Recognition warrants specificity of the cellular interaction. (*b*) There must be an exchange of humoral factors between host and invading cells. Nutritional as well as signal substances can be exchanged. Therefore, specialized transport structures on the membrane interfaces must have developed. (*c*) Stable intercellular symbiosis yields the possibility, and depends upon the development of mechanisms of control and regulation of the symbiont by the host cell. Because the membranes of both symbiotic TNC partner cells are crucially involved in the intercellular interactions, we proceeded to characterize these membranes ultrastructurally and serologically.

The majority of engulfed thymocytes, with the possible exception of recently divided cells, are completely surrounded by TNC membranes. The lymphoid cells are thus situated within single caveolae that are bordered by thin cytoplasmic septa lined by plasma membranes. We payed particular attention to ultrastructural features of the morphological relations between engulfed thymocytes and the surrounding TNC septum membranes. In ultrathin sections two distinct types of cell-to-cell contact can be discerned. Some thymocytes are completely surrounded by a tightly adhering TNC membrane. In most cases, however, intercellular contact is confined to circumscript areas. In freeze-etch preparates aggregates of particles were conspicuous on TNC

caveolar, but not on thymocyte membranes. These aggregates cannot be artificially caused by temperature decrease or by the application of cryoprotectants (39), as the cells were fixed immediately after their isolation, before freeze-etch procedures were initiated. Judging from their size and their membrane distribution, these membrane-particle aggregations might be identical with the circumscript intercellular contact areas observed in ultrathin sections. They resemble junctional membrane specializations involved in cell-to-cell communications and anchoring (13). Junction formation by lymphoid cells is not an unusual phenomenon. Various types of lymphocyte junctions have been described in several experimental situations, which all included intercellular recognition events. B-type gap junctions were recently demonstrated between phytohemagglutinin-stimulated rabbit lymphocytes (40), and septate-like junctions were seen in preparations of antigen-stimulated mouse lymphocytes (41). In addition, Sellin et al. (42) observed functional connections between specific killer T lymphocytes and relevant nonlymphoid target cells.

Apart from the probable intercellular junctional structures, TNC caveolar membranes contain macromolecular specializations, which may be of relevance for the interaction between TNC and the engulfed lymphocytes. An interesting finding in freeze-etch preparates were particles arrayed in highly regular, textured patterns on caveolar TNC membranes. Morphologically identical square array structures have been found on the extrasynaptic portions of excitable cell membranes (43), and, on plasma membranes of astrocytes (44), kidney tubular epithelium cells (45), and intestinal epithelium (13). It was suggested that these membrane specializations could be involved in membrane ion-exchange processes, possibly as components of ATPase systems (43). As another regular feature of TNC caveolar membranes, we observed membrane invaginations that may reflect micropinocytosis or, alternatively, secretory events. Although functional analyses are required to establish the exact intercellular interactions, it appears attractive to assume, that all three types of TNC caveolar membrane specializations are involved in interactions between TNC and engulfed thymocytes. The junction-like particle aggregations might be involved in establishing direct cell-to-cell contact, and, thus, in intercellular recognition. The particle square arrays and endo/exocytotic vesicles could be structures essential for determining the humoral microenvironment within the TNC caveolae.

Interpretation of semiquantitative immunofluorescent data that rely on monoclonal antibodies as markers requires critical evaluation. Binding of monoclonal antibodies by membrane determinants may be strongly affected by factors other than determinant density. The affinity of the antibodies, or the accessibility of the binding determinants on the membrane may be of relevance. Because, in the cases of H-2K/D and I-A-region antigens, we used several monoclonals directed against several different determinants of two different haplotypes, and because all antibodies behaved concordantly, it appears safe to conclude that, indeed, the high fluorescence caused by anti-I-A-antibody binding reflects a higher determinant density than the one of H-2K/D antigens tested. It is, however, more difficult to interpret the relatively low fluorescence as a result of treatment with one anti-I-E/C monoclonal antibody. This can, indeed, be a property of that particular antibody, as discussed above.

Alternatively, it is possible, that (*a*) I-E/C-region antigens are, indeed, expressed at lower density on TNC membranes than I-A antigens, or (*b*) that I-E/C-region antigens are more diverse than I-A antigens, so that within one given haplotype one monoclonal

antigen only detects one fraction among all possible determinants. In fact, it should be noted that I-E/C determinants are now believed to be controlled by more than one I-region locus (46, 47).

The demonstration of I-region-associated antigens on nonlymphoid components of the thymus may be of some importance. In contrast to the ubiquitous classical K/D histocompatibility antigens, Ia determinants were originally thought to display a marked tissue restriction, expressed only on lymphoid cells, macrophages, and sperms (15). Detection of Ia-like antigens outside the immune system was mostly restricted to macrophage-like cells, such as the Langerhans cells of the skin (48, 49). Recently, several reports were published that demonstrated Ia-like antigens within the thymic stroma (50–52). These reports made use of xenogeneic antisera, and the exact nature of the thymic cells expressing Ia antigens was not determined. This is particularly important, as it is known that the thymus contains a large proportion of macrophages (53), which do express Ia antigens. That indeed dendritic cells of the thymic reticulum do express Ia antigens was recently suggested by Rouse et al. (54), who demonstrated characteristic distribution patterns of anti-I-region antibodies on frozen sections of mouse thymuses. However, also in this study the light microscopic technique applied did not allow to definitely determine the exact cellular nature of the stained stromal elements.

Conclusion. As outlined above, the unique intracellular location of lymphocytes within epithelial TNCs reminds one of interspecies intracellular symbioses. The rules governing these xenogeneic cellular complexes should obviously also hold true when both cell partners are components of the same organism. In the case of TNC formation, it is tempting to speculate that invasion of specialized thymic epithelial cells by migratory lymphoid elements represents some key event in T lymphocyte differentiation. Recent experimental evidence suggests that T cell differentiation involves selection processes as well as cell differentiation. In the thymus, lymphocyte precursor clones are thought to expand, which can recognize and respond against determinants of the self-type MHC (2–5). The relevant determinants were postulated to be expressed on thymic epithelial cells. Jerne et al. (55, 56) has speculated that interaction of lymphoid cells with MHC determinants leads to lymphocyte proliferation, which, by involving mutational events, may lead to generation of lymphocyte diversity. These views could be reconciled with the morphological and membrane antigen properties of TNC. Indeed, TNC express high doses of all MHC determinants known to play roles in lymphocyte recognition and interaction. Recognition of TNC MHC antigens by primitive lymphoid cells could be a first recognition step leading to selective immigration into TNC. The high mitotic rate of internalized lymphocytes could be the consequence of autostimulation of these cells. In any event, intracellular proliferation may render the lymphocytes susceptible to inductive stimuli provided by the intracellular microenvironment. The unusual macromolecular specializations of the caveolar TNC membranes may well support this view.

Summary

We describe a new cellular component of normal mouse thymuses, which is isolated by fractionated trypsin dissociation of minced thymus tissue followed by repeated unit gravity sedimentation. These cells are of unusually large size, with diameters of 30 μm and more. They represent cellular complexes of single large cells filled with

high numbers of lymphoid cells. The majority of the engulfed lymphoid cells is not only fully intact, as judged by morphological criteria, but, moreover, includes a high porportion of mitotic figures. Electron microscopic investigations reveal the epithelial character of the large thymic nurse cells (TNC). The peripherally situated cytoplasmic tonofilament streams, and characteristic vacuoles filled with coarse, unidentified material, closely resemble cytoplasmic organelles found in the cortical reticuloepithelial cells described *in situ*.

The internalized lymphocytes are located within caveolae lined by plasma membranes. These TNC caveolae are completely sequestered, and have lost any communication with the extracellular space, as demonstrated by the inability of an electron-dense marker, cationized ferritin, to diffuse into the perilymphocytic clefts.

The structural interactions between the membranes of the engulfed thymocytes with the surrounding TNC caveolar membranes were investigated both in ultrathin sections and in freeze-etch preparates. Two distinct contact types between both membranes were discerned: (*a*) complete, close contact along the entire lymphocyte circumference, and (*b*) more frequently, contact restricted to discrete, localized areas. Judging from their size and distribution, the localized contacts could correspond particle aggregates of freeze-etch preparates, which morphologically resemble certain stages of gap junction. Furthermore, we regularly found square arrays of particles of uniform size, which so far have been thought to be typical for cell membranes actively engaged in ion exchange. Tight junction-like particle arrays, which were present on TNC outer membranes, and probably represented disrupted contacts between adjacent TNC in the intact tissue, could not be found on caveolar or lymphocyte membranes. Finally, one of the most conspicuous specializations of the TNC caveolar membrane were membrane invaginations, which were arranged mainly in groups, and which probably reflect endo- or exocytotoxic events.

We investigated the surface antigen phenotype of TNC by indirect immunofluorescence, with monoclonal antibodies against determinants of H-2-complex subregions as well as against lymphocyte differentiation markers. Semiquantification was reached with flow cytofluorimetry, followed by morphological control by fluorescence microscopy. The surface antigen formula of TNC is: Ig^-, Thy-1^-, H-$2K^{++}$, I-A^{++}, I-E/C^+, H-D^{++}, Ly-1^-, Ly-2^-, Qat-4^-, Qat-5^-, and peanut agglutinin $(PNA)^-$. Thymic macrophages, which were identified by double fluorescence, with rhodamine-coupled zymosan as a phagocytosis marker, were serologically identical with TNC. Free thymocytes, in contrast, had the following antigen formula: Ig^-, Thy-1^{++}, H-$2K^{\pm}$, I-A^-, I-E/C^-, H-$2D^{\pm}$, Ly-$1^{\pm}$, Ly-2^+, Qat-4^-, Qat-5^-, and PNA^+.

The unprecedented finding of high numbers of dividing thymocytes sojourning within thymic epithelial cells, and the particular specializations of the TNC caveolar membranes surrounding these engulfed thymocytes is the basis of a hypothesis that postulates that an intraepithelial differentiation cycle is one essential step in intrathymic T lymphocyte generation.

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