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In Vitro Cultivation of Nonlymphoid Thymic Cells: Morphological and Immunological Characterization

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Nonlymphoid thymic elements play an important role in T-lymphocyte development, especially in the development of recognition of transplantation antigens (H-2 in the mouse). Understanding this process will require the isolation and characterization of these cells. A simple technique for the culture of an enriched population of murine thymic epithelium is described. The epithelial nature of these cells is evidenced by their morphology, electron microscopy, and keratin content. Readily distinguishable macrophages comprise a secondary population within these cultures. Antigens encoded in the I-A region of H-2 were found on 70% of thymic epithelial cells and H-2K on 30% of thymic epithelial cells. These antigens were generally present on distinct populations but doubly positive cells were observed. Thymic macrophages were found to have conventional receptors for the Fc fragment of immunoglobulin on their surface and could ingest antibody-coated sheep erythrocytes. Thymic epithelial cells did not have such Fc receptors. A striking observation was that thymic epithelial cells could bind and internalize autologous thymocytes. This selective thymocyte ingestion by thymic epithelium may have important implications in regard to processing of T-lymphocyte precursors.

INTRODUCTION

The thymus is known to be required for the normal development of one lymphocyte subset, thymus-derived or T lymphocytes. The thymus appears to function by a variety of mechanisms involving both humoral factors (1, 2) and direct contact (3, 4). Humoral factors appear to act during the later stages ("post-thymic") of T-lineage development and permit the rapid expression of surface antigens (5) and some mitogenic responsiveness (6) in relatively mature T-cell precursors. Actual cell-to-cell contact apparently is needed for early ("pre-thymic") T-cell development of full antigenic responsiveness and tolerance (6–8), as well as of self major histocompatibility complex (MHC) recognition (9–11). These functions of the thymus have been attributed to its reticuloepithelial component, largely because of histological studies showing association of lymphoblasts with "epithelial" cells (12) and because the effects of the thymus on T-cell development are radioresistant, while thymocytes are not (11).

In order to more fuly investigate the nature and role of thymic reticulum, an *in vitro* technique was developed for growth of thymic reticuloepithelial components with negligible contamination by thymocytes or fibroblasts. This paper describes this simple and reproducible technique for the culture of murine thymic

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reticuloepithelium. Evidence for the selectivity of this culture system and characterization of the two predominant cell types are presented.

MATERIALS AND METHODS

Mice. Mice of strains C57BL/6J, CBA/J, and (BALB/c × A/J)F1 were obtained from the Jackson Laboratory, Bar Harbor, Maine. Cultures were established from animals less than 3.5 weeks of age.

Cell cultures. Dulbecco's modified Eagles' medium (DMEM) (GIBCO 320-1965) supplemented with 1 m*M* sodium pyruvate (Flow), 0.15% sodium bicarbonate, 2 m*M* Hepes (GIBCO), and 1% antibiotic/antimycotic (GIBCO) was used as base medium. Base medium was changed to Click's (EHAA) (13) after 3 weeks of *in vitro* culture. Media were supplemented with fetal calf serum carefully selected for growth-promoting potential. Initial concentration was 30%, with a gradual decrease to 10% over the period of the culture. Tissue was cultured in either 25-cm^2 flasks (Falcon 3013) or 35-mm culture dishes (Falcon 3001), using one thymus per 25-cm^2 culture surface area.

Tissue preparation. Animals were anesthetized with CO_2 and thymic lobes were removed bloodlessly while still perfused. Organs were placed in cold phosphate-buffered saline without calcium and magnesium (PBS-D). Tissue was finely minced with a scalpel blade and transferred to 15-ml conical centrifuge tubes (Falcon 2095). Pieces were washed twice with PBS-D and unit gravity sedimentation, placed in medium, and aliquoted into plates. Explant cultures were incubated at 37°C with 5% CO_2 in air at 100% humidity. Medium was added at 48 hr, and subsequently, medium was changed twice weekly. The concentration of fetal calf serum was gradually decreased after the first 10 days of culture. Prior to histological examination specimens were fixed in 10% Formalin. Staining was done using periodic acid Schiff, Hematoxylin and eosin, Masson's trichrome, reticulum, and Giemsa stains.

Reagents. Alloantisera (Table 1), kindly provided by Dr. D. B. Murphy, Yale University, were used at a 1:10 dilution. The monoclonal antibodies used are shown in Table 2. Monoclonal antibodies to I-A^k were biotin conjugated with the assistance of Dr. G. M. Iverson (14). Monoclonal antibodies were used at a dilution of 1:20 when conjugated, and 1:100 unconjugated, and were tested for specificity on peripheral lymphocytes.

The secondary stain, goat anti-mouse immunoglobulin antibody (GaMIg), was fluorescein or rhodamine conjugated (15) and used at a dilution of 1:20. Mouse immunoglobulin (MIg) was heat aggregated (16), fluorescein conjugated (15), and used immediately. Biotin-conjugated material was secondarily stained with avidin–rhodamine (Vector) at a 1:50 dilution.

TABLE 1

ALLOANTISERA TESTED BY FLUORESCENCE TECHNIQUES UPON THYMIC EPITHELIAL CULTURES

Immunization	Specificity
(A.TL × C3H.0L)F1αC3H/HeJ	H-2K^k
A.THαA.TL	Ia^k

TABLE 2
MONOCLONAL ANTIBODIES TESTED BY FLUORESCENCE TECHNIQUES UPON THYMIC EPITHELIAL CULTURES

Monoclonal antibody	Source	Specificity
10.2.16	Herzenberg (Stanford)	I-A^k(Ia-2)
11.5	Herzenberg	I-A^k
11.4.1	Herzenberg	K^k
Y-25	Lerner (Yale)	K^b

Cell surface immunofluorescence. Cultures were stained for 30 min and washed several times with medium between reactants. Dilution and washing medium was DMEM with 0.1% ovalbumin with or without 0.1% sodium azide. Tissue was mounted unfixed, in PVA–glycine. Double staining was achieved using biotin-conjugated antibody secondarily stained with avidin–rhodamine and a different antibody secondarily stained with fluoresceinated goat anti-mouse antiserum. Cultures were allowed to rest for 2 hr between each set of stainings. Order of staining did not affect results.

Cytoplasmic immunofluorescence. Keratin studies were kindly performed by Dr. Yuspa, Dr. Stanley, Dr. Poirer, *et al.* at the National Cancer Institute, Bethesda, Maryland. Techniques for fluorescence, for production of rabbit anti-keratin antisera (17), and for quantitative radioimmunoassay for mouse keratin proteins (18) have been previously reported by them. Specificity controls included negative staining on fibroblasts.

Electron microscopy. Cultures were processed for electron microscopy by a routine technique (19), with fixation being done on attached monolayers. The plastic container (e.g., petri dish) was then cracked from the mounted, intact specimen. An alternative technique was to grow the culture on a sterile Epon substrate and fix similarly *in situ.* Transmission and scanning electron microscopy were kindly performed by Margaret Koonce, Yale University.

Functional assays. Sheep red blood cells (SRC) were washed three times with PBS-D and used directly or incubated with a subagglutinating titer of mouse anti-SRC antiserum for 1 hr at 37°C. SRC or antibody-coated SRC were then washed and incubated with thymic cultures for 1 hr at 37°C. Tissue was washed and fixed with 10% Formalin prior to staining.

Ingestive abilities of the cells in culture were studied with the following material being presented to them; latex particles, SRC, antibody-coated SRC, syngeneic thymocytes, and syngeneic mature T lymphocytes. Actual internalization of SRC was proven by hypotonic lysis of all free SRC. Thymocytes were added at 10^6–10^7 per culture dish and incubated at 37°C for various periods. The unbound cells were washed out and the cultures were fixed and stained.

RESULTS

The technique for culture of nonlymphoid thymic elements described under Materials and Methods was adapted from that of Pyke and Gelfand (20) for culture

of human thymic epithelium. Little growth is observed for the first 48 hr due to overwhelming thymocyte death. Early attachment is seen at the sites of explants after 48 hr. Rapid growth of attached cells and virtually total thymocyte death occur within 1 week. Epithelial cell growth is confluent and maximal at 20 days, with fibroblast contamination being less than 5%. Use of either single-cell suspensions or older thymic donors gives increased fibroblast contamination and overgrowth. Without selective splitting, even optimal cultures become overgrown with fibroblasts by 8 weeks. For the present studies, cultures were analyzed between 2 and 5 weeks after establishment.

Two types of cells predominate in these cultures, as shown in Figs. 1 and 2. One is a large, flattened cell with long processes, a large nucleus, and multiple nucleoli. Data presented below support the conclusion that this is a thymic epithelial cell. The other cell is smaller, more rounded, and appears to be a typical macrophage. This paper describes the properties of these two cell types in culture.

The Epithelial Nature of the Large Cell Type

Several pieces of evidence support the conclusion that the larger cell type in these cultures is epithelial. By transmission electron microscopy (not shown), such cells were observed to contain tonofilaments. While pictures suggestive of tight junctions were also obtained, the difficulty in sectioning the thin monolayers made it impossible to demonstrate these structures conclusively. The most direct evidence for the epithelial nature of these cells came from two different mea-

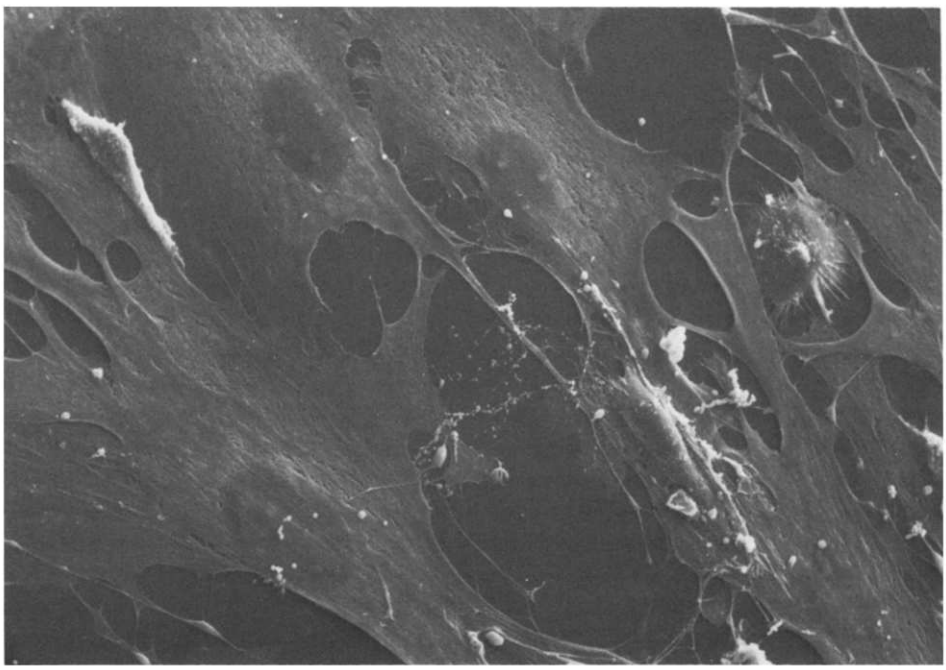

FIG. 1. Scanning electron micrograph of a thymic epithelial cell. Note the large nuclei with well defined nucleoli projected above the cytoplasmic plane.

FIG. 2. Light micrograph of a Giemsa-stained explant showing dense ingestion and degradation of antibody-coated SRC by thymic macrophages (arrowhead), as well as rare internalization of intact cells by epithelium (arrow). Free SRC have been removed by hypotonic lysis. (225 × .)

surements of keratin in their cytoplasm. The first involved immunofluorescence, as shown in Fig. 3. This staining was perinuclear and cytoplasmic and was specific for the flattened cells. A quantitative evaluation, (Table 3), showed that cultures of thymic nonlymphoid elements were enriched for keratin, as compared to whole thymus or cultures of fibroblasts. Thus, the major cell appeared to be epithelial in nature and will be referred to as a thymic epithelial (TE) cell.

Macrophages Are a Secondary Cell Type in These Cultures

As shown in Fig. 2, the other cell found in cultures of nonlymphoid thymus appeared to be a macrophage. These cells contained numerous vesicles upon electron microscopic examination. They bound large numbers of antibody-coated erythrocytes and within 2 hr ingested large numbers of these bound erythrocytes. Thus, these cells were Fc receptor positive and phagocytic. They will be referred to as macrophages in subsequent discussion. The Fc receptors on these cells make characterization of the surface antigens of these cells difficult; the subsequent sections will focus on the surface antigens expressed by the TE cells.

Cell Surface Antigens Detected on Thymic Epithelial Cells

A major postulated role of TE cells is to induce development of thymocytes bearing receptors specific for self (i.e., thymic) MHC-encoded antigens. Thus, it was critical to evaluate expression of these antigens on cultured TE cells. Studies were carried out using both conventional alloantisera and monoclonal antibodies.

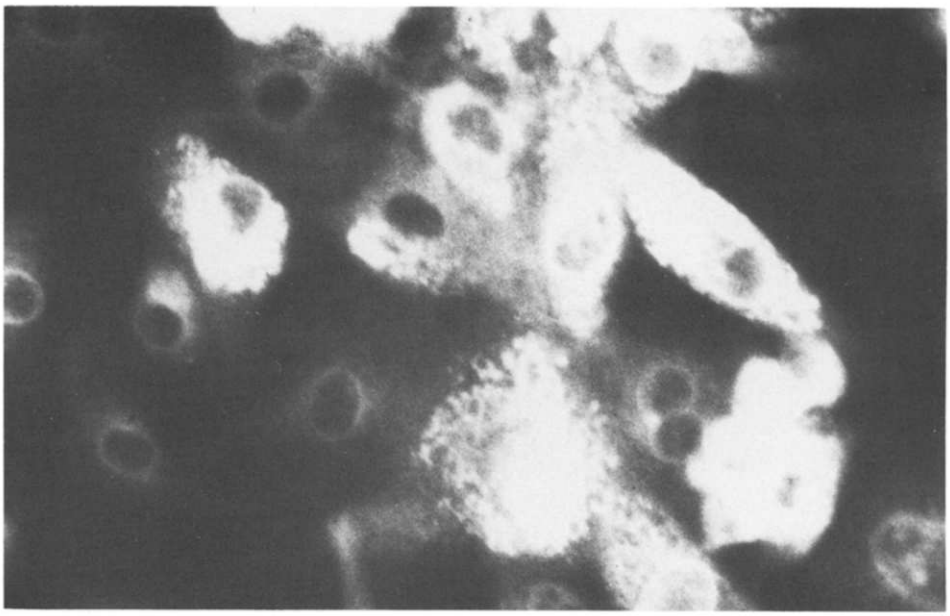

FIG. 3. Fluorescence micrograph of thymic epithelial cells stained with antikeratin antibody. Note the bright granular and perinuclear pattern. (90×.)

The results are summarized in Table 4. It can be seen that both conventional antisera and monoclonal antibodies detected products of the H-2K and H-2I regions of the murine MHC on these cells. The appearance of this staining is shown in Fig. 4. Specificity was confirmed for all reagents (Table 4). Interestingly, about 70% of the TE cells stained strongly with anti-Ia reagents, while only about 30% stained strongly with anti-H-2K reagents. In double immunofluorescence experiments, occasional cells were observed that stained brightly with both anti-Ia and anti-H-2K, but in general, one or the other product was predominantly expressed on individual TE cells.

In order to confirm that these MHC gene products were endogenously synthesized by TE cells, several criteria were applied. First, staining was stable over the period in culture (2 to 6 weeks), although virtually all thymocytes were lost

TABLE 3
KERATIN CONTENT OF VARIOUS MURINE CELLS

Source	Protein (μg/ml)	μg Keratin/mg protein[a]
Cultured thymic reticuloepithelial cells		
Sample 1	0.50	2.8
Sample 2	0.44	2.4
Fibroblasts	0.80	0.8
Fresh thymi (pooled)	1.73	0.4
Epidermal cells	0.95	18.4

[a] Keratin content was determined by quantitative radioimmunoassay (see Materials and Methods).

TABLE 4

STAINING OF TE CELLS WITH ALLOANTISERA AND MONOCLONAL ANTIBODIES SPECIFIC FOR MHC GENE PRODUCTS[a]

Source of thymic epithelium			Antibody tested					
	MHC genotype		Alloantisera		Monoclonal antibodies			
Strain	H-2K	Ia	Anti-H-2K^k	Anti-Ia^k	10.2.16(I-A^k)	11.5(I-A^k)	11-4.1(K^k)	Y-25(K^b)
C57BL/6J	b	b	0	0	–	–	–	+
CBA/J	k	k	+	+	+	+	+	NT
(BALB/cxA/J)F1	kxd	kxd	+	+	+	+	–	–

[a] +, Specific staining observed; –, no staining observed; NT, not tested. Staining with secondary stains alone was negative for all of the above strains.

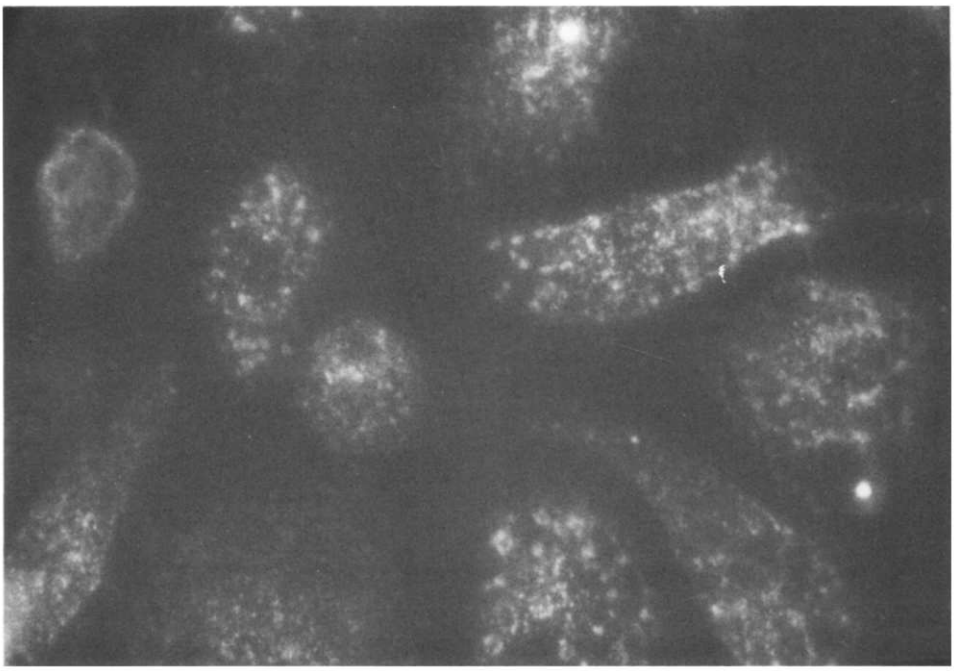

FIG. 4. Fluorescence micrograph of TE stained with monoclonal antibody Y-25 to H-2K^b. TE was derived from stain C57BL/6J (H-2^b). Note the beaded pattern of staining along entire surface of epithelial cells. (375 ×.)

from the cultures after 1 to 2 weeks. Second, the pattern of antigen expression was selective for individual cells, a finding inconsistent with passive uptake. Third, culturing the TE cells with fresh, allogeneic thymocytes did not lead to acquisition of allogeneic MHC gene products by the TE cells (Table 5). Thus, the evidence is consistent with endogenous synthesis of MHC gene products by the TE cells. While it can not be ruled out that these molecules derive from the macrophages, the selective expression on individual TE cells is against this possibility as well.

Evidence for expression of the thymocyte differentiation alloantigens Thy-1, Lyt-1, and Lyt-2 was also obtained; however, in this instance, passive acquisition

TABLE 5

TEST FOR PASSIVE ACQUISITION OF SURFACE ANTIGENS BY THYMIC EPITHELIUM[a]

Thymocyte	TE	Specificity of monoclonal antibody	Result[b]
CBA/J(H-2^k)	C57BL/6J(H-2^b)	K^k	–
C57BL/6J(H-2^b)	CBA/J(H-2^k)	K^b	–

[a] Thymic cultures were incubated with thymocytes at 37°C, 5% CO_2 for 20 hr. Cultures were washed free of thymocytes and stained with monoclonal antibody at a 1:100 dilution for 30 min at room temperature. They were then rewashed and secondarily stained with fluorescein-conjugated goat anti-mouse antibody diluted 1:20 with staining buffer.

[b] –, no staining observed.

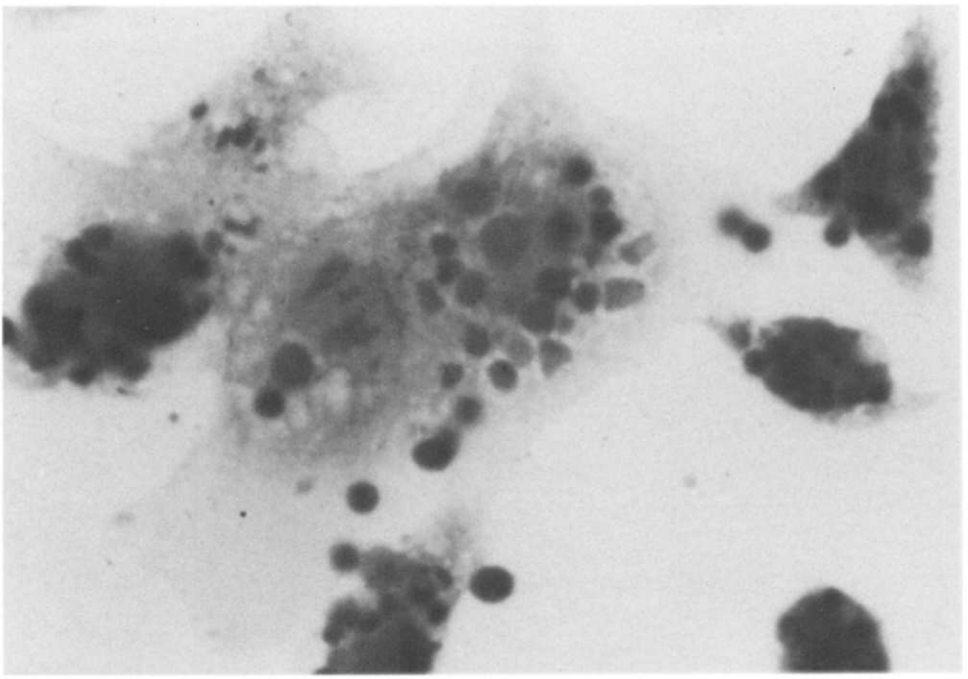

FIG. 5. Light micrograph of Giemsa-stained TE after 20 hr of incubation with syngeneic thymocytes. Free thymocytes were removed by gentle serial washings. Note the large number of cells intact within the cytoplasm of epithelial cells. (~600 ×.)

from exogenously added allogeneic thymocytes was observed, albeit with a totally different staining pattern. Thus, expression of these antigens by TE cells, while interesting, can not be clearly demonstrated to involve endogenous synthesis at this time (data not shown).

Ingestion of Syngeneic Thymocytes

As stated previously, the macrophages in these cultures had cell surface Fc receptors and rapidly ingested antibody-coated erythrocytes. Thymic epithelial cells did not bind significant numbers of antibody-coated erythrocytes and only rarely and very slowly ingested these particles (Fig. 2). However, when confronted with syngeneic thymocytes, both macrophages and the majority (about 80%) of the TE cells ingested large numbers of these cells. Figure 5 shows this phenomenon after 20 hr of culture. The syngeneic thymocytes persisted in the cytoplasm of the cultured TE cells for at least 24 hr after washing out unbound cells, apparently in viable form (Fig. 6), while those ingested by the macrophages rapidly break down. Mature lymphocytes are taken up poorly, if at all, by either cell type.

DISCUSSION

The thymus plays a critical role in the development of bone marrow-derived precursor cells into mature T lymphocytes. Although much of the *in vivo* investigation of the role of the thymus has been done in mice, cultures of murine

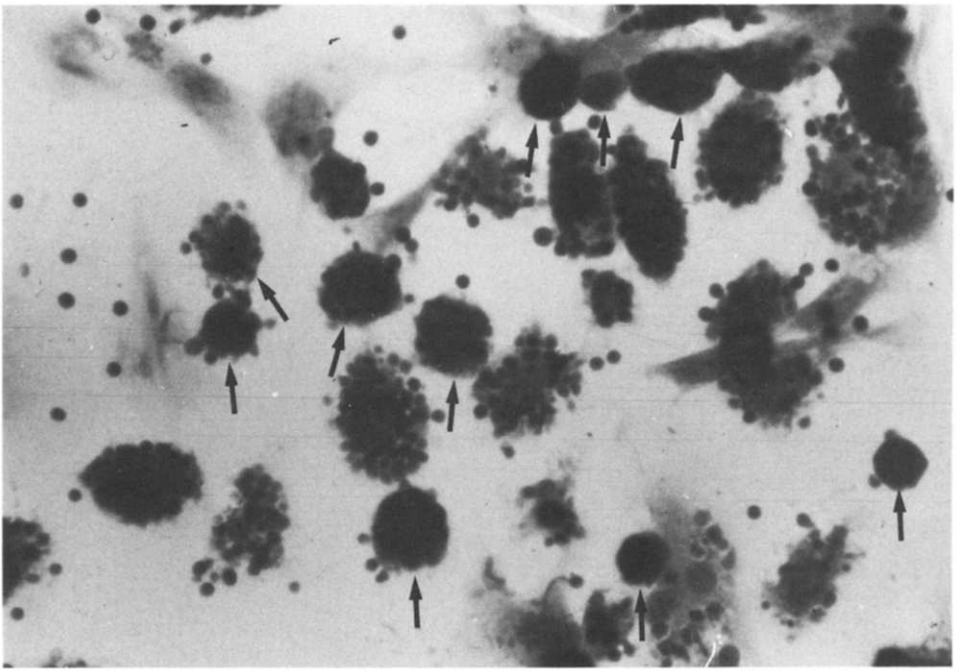

FIG. 6. Light micrograph of Giemsa-stained TE cultured 24 hr after removing added syngeneic thymocytes. Ingested and degraded thymocytes cause macrophages (arrows) to stain darkly. Intact thymocytes are seen within TE cells. (~150×.)

thymic epithelium have been difficult to obtain. Despite this, a number of authors have developed selective techniques for examining the thymic structure and/or function *in vitro* (21–32). The technique described here has proven to be as successful as those incorporating more complicated procedures and permits selective cell growth with minimal expenditure of time and materials. It has been applied successfully to a broad range of mouse strains, permitting comparative analysis of congenic lines. One critical variable may be the fetal calf serum. Only certain lots of serum favor TE cell growth when used in a high concentration.

Strong evidence for the predominant growth of epithelial cells in these cultures has been obtained using histological, electron microscopic, and biochemical (i.e., keratin content) criteria. Thymic macrophages also persist within this culture system, as they clearly do in others as well. The role of macrophages in T-cell ontogeny remains to be examined, but is especially worthy of consideration given the current confusion in attempting to analyze data obtained in bone marrow chimeras (33).

Besides describing the ease with which this technique yields selective growth of TE cells, we have made two very interesting observations concerning these cells. One is the apparently endogenous production of MHC-encoded antigens by TE cells. This is particularly important in the case of Ia glycoproteins. These molecules are expressed selectively on cells predominantly involved in immune function: B lymphocytes, macrophages, dendritic cells, and Langerhans cells in

the skin. These glycoproteins are recognized by T lymphocytes as critical markers of self in cell–cell interactions during responses to exogenous antigen. Evidence from thymic chimeras suggests that a nonlymphoid cell in the thymus selects those thymocytes having specificity for these self-Ia antigens for expansion and peripheralization (34). For TE cells to play this role, they must be able to express Ia glycoproteins as do the cells in our culture system. The relatively poor expression of the H-2K-encoded glycoproteins on these cultured TE cells (only 30% stained brightly with anti-H-2K reagents) is also surprising, since all murine cells are thought to carry H-2K products. Thymic selection for self-H-2K-recognizing T cells also operates in most experimental systems. However, the rich representation of Ia-bearing TE cells and the relatively poor representation of H-2K products on these cells are consistent with earlier studies on the expression of these antigens in frozen sections of thymus carried out by Weissman *et al.* (35). Although thymic macrophages have also been shown to express Ia antigens (36), the very distinctive morphology of the dendritic epithelial cells, as well as their lack of Fc receptors, makes the definition of these cultured thymic epithelial cells as Ia positive relatively simple and conclusive.

The postulated role of the thymic epithelial cells in selecting self-MHC-recognizing T cells implies an interaction between the thymic epithelial cell and the thymocyte, leading to activation of those thymocytes bearing appropriate self-recognizing receptors. The striking observation that the cultured TE in this system ingest syngeneic thymocytes is of considerable interest in this regard. It is not possible at the present time to determine if the ingested cells, which appear to be viable, are different from those not ingested, nor are studies with allogeneic thymocytes useful in this instance, as these are also ingested (J. Jason, unpublished observations). While this latter observation would seem to argue against involvement of specific self-MHC recognition in the ingestion phenomenon, this is not necessarily the case. One could easily argue that thymocytes represent an immature, and hence unselected, population of T lymphocytes, bearing a random representation of receptors specific for all MHC gene products. Alternatively, the allogeneic thymocytes ingested could be destined to become alloreactive T lymphocytes and would thus bear receptors specific for the MHC gene products of nonself TE. This process needs to be studied either with mature T lymphocytes, which should bear predominantly anti-self MHC receptors, or by recovering F1 thymocytes ingested by parental TE cells and testing their ability to recognize either parent's MHC antigens as self. Such experiments should determine the specificity of this interaction.

If ingestion reflects self-MHC recognition, then one could explain the failure of mature T cells to be ingested by TE cells in several ways. It could be due to changes in the surface properties of mature T cells, such as the acquisition of sialic acid (37), or it could reflect a change in the expression of self-MHC recognition units on such cells. The apparent ability of TE cells to induce the clonal expansion of thymocytes specific for self-MHC determinants, while peripheral T cells clearly possessing self-MHC-recognizing ability are not activated by the same self determinants, is consistent with our observations on thymocyte ingestion. Therefore, this process deserves further study. This process of thymocyte

ingestion may represent an *in vitro* correlate of the similar morphological form known as the thymic nurse cell characterized by Wekerle and Ketelson (38).

In summary, the present system allows the rapid and simple selective culture of nontransformed thymic epithelial cells, contaminated with some macrophages and few fibroblasts. These TE cells have cell surface antigens encoded in the MHC and interact in an interesting way with added thymocytes, consistent with the postulated role of TE cells in the induction of the T-cell repertoire.

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