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Cloning of Human Thymic Subcapsular Cortex Epithelial Cells by SV40 ori$^-$ Transfection

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A. Introduction

Critical steps in the early differentiation of T lymphocytes occur within the thymus. Bone-marrow derived cells migrating into this organ undergo extensive proliferation, clonal rearrangement of antigen receptor genes and an associated immunological "education" involving tolerance to self-antigens and positive selection for antigen recognition in association with self-MHC (Zinkernagel 1978; Haynes 1984; Rothenberg and Lugo 1985; Lo and Sprent 1986). The control of this complex process of commitment, expansion, clonal diversification, selection and maturation is not understood, but almost certainly involves selective interactions with distinct elements of the thymic stromal environment and diffusable regulators, thymic "hormones" or growth factors. The nonlymphoid stromal structure of thymic tissue consists of different types of epithelial cells derived from pharyngeal pouch endoderm or brachial cleft ectoderm, mesenchymal cells and bone-marrow derived histiocytes or interdigitating macrophages (Le Douarin et al. 1984; van de Wijngaert et al. 1984; Haynes 1984; Janossy et al. 1986).

Unravelling the interactions between developing T cells and microenvironmental components requires cell culture techniques similar to those established for bone marrow myelopoiesis (Dexter 1982) and methods for isolating and cloning individual stromal cell types.

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Some success has been reported in culturing rodent and human thymic cells characterized as epithelial by desmosomes, tonofilaments, cytokeratins or membrane antigens and culture supernatants from such cells can regulate T-cell phenotype or immunological function (Itoh et al. 1981; Beardsley et al. 1983; Glimcher et al. 1984; Singer et al. 1985). However, cloned lines representing distinct subtypes of thymic epithelia have not so far been established.

The subcapsular epithelium of thymus has a distinctive structure and is probably the first site of interaction with migrating T-cell precursors (van de Wijngaert et al. 1984; Janossy et al. 1986; Haynes et al. 1984). We have sought to isolate and grow these epithelial cells by a combination of selective culture conditions and gene transfection techniques. We report here the successful establishment of two such cell lines which retain phenotypic properties of subcapsular epithelium and express some endocrine and growth-regulating functions.

B. Materials and Methods

I. Primary Epithelial Cell Culture

Thymic epithelial cells were derived from a 16-week-old human fetus obtained with the consent of the Ethics Committees of the Institute of Cancer Research and the Royal Marsden Hospital, London. Thymic tissue was minced and grown in a 25-cm^2 tissue culture flask in Dulbecco's modified Eagle's medium (DMEM; Gibco Biocult, Scotland),

supplemented with 5% (v/v) fetal calf serum (FCS; Sera Lab, England), 1.8×10^{-4} *M* adenine (Sigma, England), 5 μg/ml insulin (Sigma, England), 10^{-10} *M* cholera toxin (Sigma, England), 0.4 μg/ml hydrocortisone (Sigma, England) and 20 ng/ml epidermal growth factor (BRL). This AICHE-FCS medium was completely replaced each week.

II. Transfection
of Primary Epithelial Cells

Epithelial enriched cultures were transfected 3 weeks after initiating their growth using calcium phosphate. Cells were transformed by cotransfection with the SV40 ori$^-$ mutant 6-1 (Gluzman et al. 1980; Nagata et al. 1983) and PSV-2 neo. As controls, cells were either transfected with PSV-2 neo alone or were grown as primary epithelial cells as described above. One month after transfection, cells were selected with G418 (Gibco Biocult, Scotland) at a concentration of 1 μg/ml. The nontransfected cells were sensitive to these conditions and could not be maintained in culture for more than 4 weeks. Two colonies (SM1 and SM2) were obtained from the SV40 ori$^-$ and PSV-2 neo cotransfected cultures and these reached confluence 3 weeks after transfection. These cells were passaged by trypsinization and replated at $1–2 \times 10^5$ cells per 25-cm^2 tissue culture flask.

C. Results and Discussion

I. Transformation
of Thymic Epithelial Cells

Cells in the primary culture were morphologically heterogeneous and consisted of dense polygonal cell islands surrounded by spindle-shaped, elongated fibroblasts. Cytokeratin staining using the LE61 monoclonal antibody indicated that these cultures comprised 60%–70% epithelial cells. Cotransfection of such cells with SV40 ori$^-$ and PSV-2 neo provided two transformed colonies, SM1 and SM2. SM2 cells appeared to be morphologically homogeneous, whereas the SM1 consisted of two morphologically distinct cell types. The latter comprised small, elongated fibroblastoid cells and polygonal cells. SM1 cells were cloned by limiting dilution in the presence of a primary thymic stromal feeder layer which had been subjected to 5000 rads *X*-irradiation. Two weeks later two morphologically distinct subclones, SM1.1 and SM1.9, were selected by their G418 resistance. The former contained fibroblastoid-like cells, while the latter comprised the polygonal cells.

Both SM1 and SM2 cells had desmosomes and tonofilaments characteristic of epithelial cells (Fig. 1). The subclones SM-1 and SM1-9 also showed tonofilaments and desmosomes.

II. Immunocytochemical Characterization

Transfection of cells with SV40 ori$^-$ was confirmed by the presence of nuclear large T antigen in the original SM1 and SM2 clones (Fig. 1; see Table 1 for summary). The epithelial nature of the original clones and of the SM1.1 and 1.9 subclones was shown by reactivity of intermediate filaments with cytokeratin antibodies (Lane 1982; Cooper et al. 1985; Chang 1986; see Fig. 1). Several monoclonal antibodies which had been characterized on thymic tissue sections (Haynes 1984; Janossy et al. 1986) were used to define the type of epithelial cells isolated (Table 1). Thy-1 human antibody reacted strongly with the original clones and the subclones as assessed by fluorescence microscopy and the FACS. Thy-1 has been shown previously to specifically identify the thymic subcapsular cortex epithelium (Ritter et al. 1981; Janossy et al. 1986). Thirty percent of cells in the original SM1 and SM2 clones were also positive for the A_2B_5 antigen, which is expressed on both the subcapsular cortex and medullary epithelia (Haynes et al. 1984). In addition, the RFD4 antibody, which recognizes the subcapsular cortex epithelium in pediatric thymus (Janossy et al. 1986) and the natural killer (NK) cell marker, Leu7, which occurs on the subcapsular cortex of the fetal thymus (Janossy et al. 1986) were found to react weakly with the isolated epithelial cells. These findings indicated that SM1 and SM2

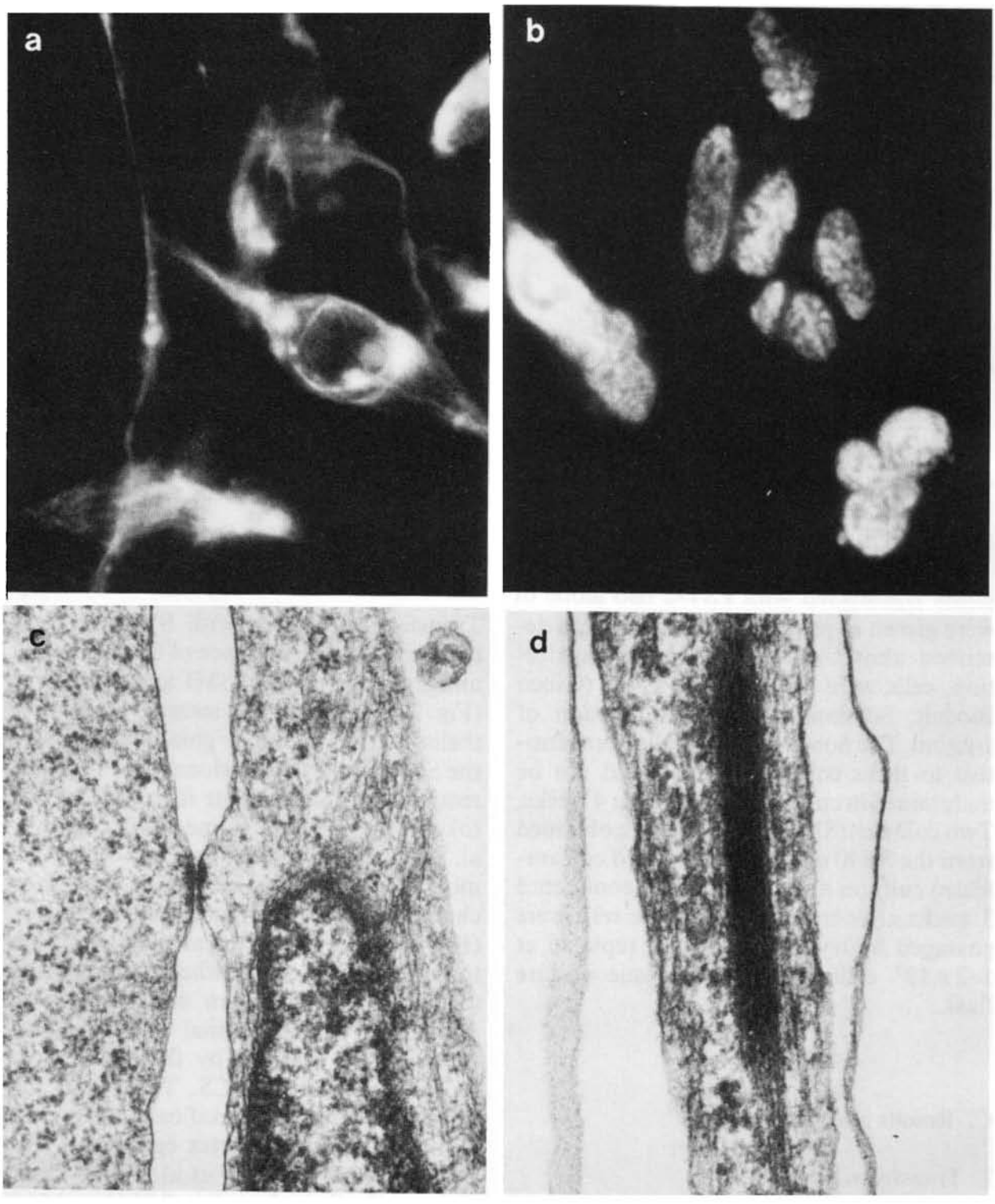

Fig. 1 a–d. Phenotypic characteristics of SV40-transformed human thymic subcapsular cells. **a** Immunofluorescent staining with monoclonal antibody (Le61) to cytokeratin. **b** Immunofluorescent staining for nuclear SV40 large T antigen. **c** Desmosome. **d** Tonofilaments

were derived from subcapsular cortex epithelia and retained the antigenic phenotype characteristic of this tissue.

SV40 transformed subcapsular epithelial cells expressed HLA-AB determinants, but not HLA-DR, HLA-DQ, nor HLA-DP. Lymphoid cell markers which were defined by CD1, 2, 3, 4, 5, 7, 8, 10 specific monoclonal antibodies (Reinherz et al. 1986) could not be identified on any of the clones. There was also no reactivity with the 3.9 monoclonal antibody which recognizes thymic monocytes and interdigitating dendritic cells (Janossy et al. 1986).

Table 1. Phenotypic characterization of SV40 ori$^-$ transformed thymic epithelial cells and their sublines

Clone	Positive cells (%)									
	Le61 Cyto-keratin[a]	SV40-T	Thy-1	A2B5	Chro-mo-granin	HLA-A, B	Trans-ferrin receptor	Vimen-tin	Leu7	RFD4
SM1	100	100	100	26	100	100	100	100	10	10
SM1-1	100	100	100	76						
SM1-9	100	100	100	83						
SM2	100	100	100	35	100	100	100	100	10	10

[a] Le61 recognizes a 40–45 daltons. Keratin components of intermediate filaments found predominantly in simple, nonstratified epithelia (Lane 1982; Cooper et al. 1985).
The clones did not react with antibodies to HLA-DR, CD1, CD2, CD3, CD4, CD5, CD7, CD8, CD10, Factor VIII, 3.9 (anti-monocyte), BI.3C5 (antihuman haemopoietic progenitor cell antigen, HPC-1), Desmin and HTLV-I P19.

III. Endocrine Characteristics of Subcapsular Cortex Epithelial Cells

The presence of chromogranin is thought to be indicative of endocrine function within the neuroendocrine system and has recently been identified in rat thymic epithelium (Hogue-Angeletti and Hickey 1985). Chromogranin was identifiable in the isolated clones SM1 and SM2 by an chromogranin monoclonal human antibody suggesting that these cells may have an endocrine function. There was no staining with antibodies to the thymic hormones thymopoietin and thymulin (FTS).

IV. Subcapsular Cortex Epithelial Cells Support Hemopoietic Cell Growth

It was of particular interest to know whether the SV40 transformed thymic epithelial cells could influence commitment to the T-cell lineage or induce the proliferation and differentiation of immature T cells. Fetal liver, a source of hemopoietic progenitors, was therefore cultured in methyl cellulose above irradiated or nonirradiated subcapsular cortex epithelial cells. Two types of colonies formed; one consisting of large tightly packed cells and the other comprising more diffuse colonies of smaller cells. Cytochemical staining revealed that both colony types contained nonspecific esterase (α-naphthyl butyrate esterase) and acid phosphatase positive cells. Morphologically, the more diffuse colonies contained small monocytes while the tightly packed colonies appeared to be activated macrophages. The monocyte-macrophage nature of these colonies was confirmed by positive staining of the cells with anti-3.9 and anti-HLA-DR monoclonal antibodies. None of the colonies tested contained either the intracellular TdT or T3δ markers characteristic of T-cell precursors (Furley et al. 1986). This culture system therefore did not support or induce early T-cell differentiation from fetal liver precursors, but rather induced macrophage development.

Thymic subcapsular epithelial cells probably have more than one function in T-cell development. Their physical location accords with a potential role in chemotaxis of bone marrow precursors and this possibility is being assessed with the clones described here. Our data suggest that these epithelial cells may also regulate the activity of immigrating or resident macrophages and thereby indirectly modulate the proliferation or selection of immature T cells. It is likely that thymic subcapsular epithelial cells are also involved in the activation of the dividing T lymphoblasts with which they are in intimate contact in situ (Janossy et al. 1986; Singer et al. 1986; Wekerle and Ketelson 1980). Further investigation of the functional activity of the SV40 transformed thymic epithelium cell lines is in progress.

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