ESTABLISHMENT AND CHARACTERIZATION OF A THYMIC MEDULLARY
EPITHELIAL CELL CLONE

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SUMMARY

Thymic stromal cells were cultured in conditions which select for epithelial cells. These were then transformed in vitro by contact with N-methyl-N'-nitro-N-nitrosoguanidine and cloned at limit dilution. One of the clones was characterized as being of medullary origin on the basis of its reactivity with a battery of antibodies previously shown to distinguish cortical from medullary thymic epithelial cells. The importance of this clone lies in the potential it offers to delineate how various T cell subpopulations acquire their distinct markers and function within the thymus.

Key words: thymic epithelial; epithelial clone.

INTRODUCTION

While it has been well established that the thymus is responsible for T-lymphocyte maturation, the precise nature of the interactions between T-lymphocyte precursors and thymic stromal cells remains largely unexplored, with one notable exception: thymic hormone, secreted by thymic epithelial cells is known to play an essential role in the maturation of T-lymphocytes (8). There is however undisputable evidence that other equally important signals are transmitted by the thymic microenvironment to thymocytes through cell-cell contact (3). The ultimate outcome of intrathymic T-cell differentiation is a variety of T cell subsets distinguishable both by their function and by their surface markers (5).

This heterogeneity in T cells is mirrored by the more recently established heterogeneity in thymic epithelial cells. A number of monoclonal antibodies have been described which distinguish epithelial cells of the cortex from those of the medulla (10).

In order to gain an insight into the differentiation pathways of various T cell subsets, it is of capital importance to develop ways of studying the various elements of the thymic microenvironment, in isolation from each other.

In the present paper we describe a thymic epithelial cell clone (E5) possessing all the known markers of medullary epithelial cells, namely cytokeratin, present on all epithelial cells, (6) Ia, present in the thymus, mainly in the medulla, (9) and TMF, present exclusively in the medulla. ERTR5 and ERTR6 monoclonal antibodies reacting selectively with medullary epithelium in situ, stained E5 cells, while ERTR4 and ERTR7 antibodies, detecting respectively cortical epithelium and thymic reticular fibroblasts, did not stain E5 cells.

MATERIALS AND METHODS

Epithelial cell culture. Thymic stromal cells from 4-5 week old C57B1/6 mice were cultured essentially as described by Boniver et al. (2). Briefly, minced thymuses were rided of lymphocytes, trypsinized and plated into 60 mm flasks in Waymouth medium with 10% "Nuserum" (Collaborative Research, Lexington, MA, USA). After two days, the cultures were washed and MEM-d-Valine (Gibco) was added to the cells to inhibit fibroblast growth. Remaining fibroblasts were removed by gentle trypsinization as required. After two weeks, the medium was supplemented with 100 U/mL Mycostatin. This insoluble fungicidal agent is rapidly ingested by macrophages, which degenerate in a few days.

Transformation of epithelial cells. The technique developed by Steele et al., (7) for the in vitro transformation of rat tracheal epithelium was adapted to the mouse thymus. N-Methyl-N-Nitro-N-Nitrosoguanidine (MNNG) was added to three-week old cultures (10 µg/mL) and kept for 6 hours, twice, with a three day interval. Transformation began within six months.

Cloning. Cloning was done by seeding the cells at limit dilution into 96 well flat bottom plates. Selected clones were expanded and samples were cultured in slide chambers (Lab-Tek) for histochemical analysis.

Control cells. A fibroblast line (BF) from C57B1/6 embryonic mice was used as a control culture.

Antibodies. Antibodies used were guinea-pig anti cytokeratin from Miles Laboratories, Anti Ia' was a monoclonal mouse IgM from Daymar Laboratories, Toronto. Anti-TMF, a rat IgM, was prepared in our own laboratory (11) ERTR4, ERTR5, ERTR6, and ERTR7, rat monoclonals, were gifts of Dr. W. Van Ewijk. Conjugates were rabbit FITC-anti-guinea pig serum, from Miles, rabbit FITC-anti-mouse IgM from Dimension
Laboratories, Toronto, and peroxidase rabbit anti-rat Ig from Nordic Laboratories, El Toro, CA.

**Immunocytochemistry.** Immunocytochemical analysis was done on cells cultured in slide chambers either by immunofluorescence with the appropriate fluorescent or rhodamine conjugates in the case of anti-cytokeratin and anti-Ia, or by the peroxidase technique in the case of anti-TMF and ERTR4, ERTR5, ERTR6, and ERTR7.

**Immune electron microscopy.** Non fixed cells were treated in the culture flask with anti-TMF antibodies diluted 1:4 in PBS-Tween (0.05%) for 30 min. at room temperature and washed three times in PBS. Goat IgG anti rat IgM was then added in the same way, washed and followed by protein A-gold (1:10) which was incubated at 4 °C. The preparations were washed, fixed with 2.5% buffered glutaraldehyde and postfixed in osmium tetroxide. After washing, the cells were removed with a rubber policeman, pelleted, and embedded in Spurr resin. Ultrathin sections were viewed with a Philips 300 electron microscope.

**RESULTS**

**Characterization of the E5 clone.** The E5 clone was selected on the basis of its morphological homogeneity, its stability and its relatively predictable mitotic rate of 30 × per week. The cells, when grown at confluence, formed mosaic-like patterns, had a large polygonal nucleus with dispersed chromat and several nucleoli. Characteristic granules were present in the cytoplasm. At the ultrastructural level, it was apparent that the cytoplasm had well developed endoplasmic reticulum as well as polyribosomes, bundles of tonofilaments and desmosome-like structures.

**Histochemistry.** The intensity of reactivity of immunofluorescence and of immunoperoxidase with semi-confluent cell preparations was scored on an arbitrary scale of − to ++++. It is apparent (Table 1) that E5 cells gave a strong reaction with anti-cytokeratin antibodies (Fig. 1a) and with anti-Ia* antibodies (not illustrated) while BF control cells were negative with these antibodies.

**TABLE 1**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity *</th>
<th>Staining Intensity</th>
<th>E5</th>
<th>BF</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-cytokeratin</td>
<td>All epithelial cells</td>
<td>++++ +++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>anti-Ia*</td>
<td>Preferentially medulla</td>
<td>+++ +++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>anti-TMF</td>
<td>Medullary epithelia</td>
<td>+++ (+)</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>ERTR4</td>
<td>Cortical epithelium</td>
<td>+++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>ERTR5</td>
<td>Medullary epithelium</td>
<td>+++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>ERTR6</td>
<td>Medullary epithelium and lymphoid cells</td>
<td>+++ ++ (+)</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>ERTR7</td>
<td>Reticular fibroblasts</td>
<td>(+)</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

*See text for references.

Staining by immunofluorescence or immunoperoxidase is expressed in arbitrary units from − to ++++.

Anti-TMF gave a strong reaction (Fig. 1b), as well as ERTR5 (Fig. 1c) and ERTR6. ERTR4 and ERTR7 gave a virtually negative reaction with E5. In order for this semi-quantitative analysis to be valid, all antibodies were used at the dilution which gives a ++++++ reaction with their respective target cells on frozen thymic sections.

**Ultrastructural distribution of TMF on E5 cells.** Colloidal gold particles were distributed fairly homogeneously along the plasma membrane of E5 cells with however a stronger accumulation on microvilli (Fig. 1d). Since whole cells were treated with antibodies before fixation, intracytoplasmic organelles cannot be expected to be marked.

**In vivo passage.** Attempts at passaging E5 cells in vivo, either subcutaneously or intraperitoneally generally failed; the cells developed neither a solid nor an ascitic tumor and could not be recovered.

Intra-thymic injection of as few as 3000 cells, resulted in no obvious change in thymic size or histology; however epithelium from such thymuses when cultured, behaved as the original transformed cells, even after six months in vivo.

**DISCUSSION**

The E5 clone, on the basis of reactivity with anti-cytokeratin antibody, was established as being epithelial in origin. Furthermore, its reactivity with antibodies specific for medullary epithelial cells (ERTR5, ERTR6, and anti-TMF) clearly shows its medullary phenotype. This point is confirmed by the virtually negative reaction of E5 cells with ERTR7 (specific for thymic fibroblasts) and with ERTR4 (specific exclusively for thymic cortical epithelial cells).

It is interesting to note that the distribution of TMF on the plasma membrane of cultured E5 cells was somewhat different from that previously observed on medullary epithelial cells in situ: in the former it was rather homogeneous with some concentration on microvilli, while in the latter, it was found exclusively at the contact points with adjoining thymocytes (11). Direct quantitative comparison with the in situ distribution of this antigen cannot be done, since labelling in the former case was done on whole unfixed cells whereas in the latter case it was done on fixed ultrathin sections.

Other thymic epithelial cell lines have been described in the mouse and in the rat (1,4). They have been characterized by morphological criteria, reactivity to anti-cytokeratin and the induction in vitro on thymocytes (or thymocyte precursors) of some parameters of maturation. These lines, however, did not distinguish between various epithelial cell types, and it is difficult therefore to determine whether they represent one, several, or all thymic epithelial cell types.

While it has not yet been established whether there is a direct correlation between a given epithelial cell type and a specific differentiation signal transmitted to a particular thymocyte subpopulation, the heterogeneity in T cells makes this question particularly relevant. The availability of characterized epithelial cell clones such as E5, now brings the answer to this question within reach.