Thymic Epithelial Cells Provide Unique Signals for Positive Selection of CD4<sup>+</sup>CD8<sup>+</sup> Thymocytes In Vitro

By Graham Anderson, John J. T. Owen, Nel C. Moore, and Eric J. Jenkinson

From the Department of Anatomy, Medical School, University of Birmingham, Birmingham B15 2TT, UK

Summary

Using a novel system that supports positive selection in vitro, we have investigated the cellular requirements for this process by testing the ability of individual thymic and nonthymic stromal cell types to support the maturation of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes into CD4<sup>+</sup> or CD8<sup>+</sup> T cells. We show that thymic cortical epithelial cells are unique in their ability to mediate this maturation, and suggest that in addition to TCR ligation, these cells supply specific signals for positive selection. Moreover, by demonstrating positive selection on ECDI (1-ethyl-3-[3(dimethyl-aminopropyl]-carbodiimide)-fixed epithelial cells in this system, we provide direct evidence that the provision of these signals involves interactions with epithelial cell surface molecules rather than the release of soluble factors.

Materials and Methods

Mice

BALB/c mice embryos at day 14 of gestation, produced by timed matings, were used as a source of fetal material.

Antibodies

For immunomagnetic selection, the following antibodies were bound to either anti-rat or anti-mouse coated magnetic beads (Dynal, Wirral, UK) as appropriate: anti-CD3 (clone KT-3; Seralab, Crawley, Down, UK), anti-CD8 (clone YTS 169.3; Serotec, Ltd., Oxford, Oxon., UK), anti-CD45 (clone M1/69; American Type Culture Collection, Rockville, MD), anti-I<sub>α<sup>+</sup></sub> (clone MK-D6; Becton Dickinson & Co., Mountain View, CA), and anti-GQ ganglioside (clone A2BS; a gift of M. Raft, University College, London, UK). For flow cytometric analysis of thymocyte differentiation, the following reagents were used: anti-αβTCR (clone H57.597; a gift from R. Kubo, National Jewish Centre for Immunology and Respiratory Medicine, Denver, CO), anti-CD4-PE (clone GK 1.5; Becton Dickinson & Co.), anti-CD8-FITC (clone 53-6.7; Becton Dickinson & Co.), anti-hamster F(ab)2 biotin (Caltag Laboratories, San Francisco, CA), and streptavidin-APC (Becton Dickinson & Co.).

Preparation of Cell Types

Thymocytes. To obtain a population of CD4<sup>+</sup>CD8<sup>+</sup> cells not yet exposed to TCR-mediated positive selection signals in vivo, CD4<sup>+</sup>CD8<sup>+</sup> T cells were prepared from newborn thymocytes by immunomagnetic selection as described previously (10, 11).

Thymic and Nonthymic Stromal Cells. Stromal cell suspensions for incorporation into reaggregate cultures were prepared from thymic and other fetal rudiments as previously described in detail (10, 11). Briefly, dispersed suspensions of whole thymic stromal cells were prepared by trypsinization of 2%Guo-treated fetal thymus lobes. Purified MHC class II<sup>+</sup> thymic cortical epithelial cells were used in the experiments.
Flow Cytometry

Freshly purified CD4+CD8+ TCR- thymocytes and thymocytes harvested from reaggregate organ cultures by mechanical disruption were pelleted by centrifugation and incubated sequentially in 100 μl of anti-αβTCR (H57.597 tissue culture supernatant), anti-hamster F(ab')2, biotin and a mixture of CD4-PE, CD8-FITC, and streptavidin-APC. Stained cells were fixed in 1% paraformaldehyde and analysis was performed on an Elite Dual Laser machine (Coulter Electronics Inc., Hialeah, FL) with forward and side scatter gates set to exclude nonviable cells. Gates defining TCR-, TCR+, and TCR+ populations were set by comparison with adult thymocyte suspensions stained in parallel.

Results and Discussion

Thymic Epithelial Cells Provide Unique Signals for Positive Selection of CD4+CD8+ Thymocytes In Vitro. Newly generated CD4+CD8+ thymocytes have an average life span of 3–4 d, after which they die unless rescued for further maturation into single positive CD4+ or CD8+ cells by positive selection (3, 15). Thus, to examine the ability of different stromal cells to mediate positive selection, each cell type was reaggregated individually with purified CD4+CD8+ thymocytes and the cultures were analyzed for cellular content and T cell differentiation after 4 d. As shown in Fig. 1 a, viable cells (>30% of the input number) were recovered from reaggregates with purified MHC class II+ thymic epithelium. In contrast to freshly purified CD4+CD8+ TCR- thymocytes used for the reaggregate input (Fig. 2, a and b), these cells were predominantly single positive CD4+ or CD8+ cell expressing high levels of αβTCR (Fig. 1 b), indicating that positive selection had taken place. Moreover, cells recovered from these cultures were able to proliferate in response to stimulation by superantigen, indicating that they had acquired functional as well as phenotypic maturity (13, and data not shown).

In contrast to reaggregates with thymic epithelial cells, very few viable cells were recovered from cultures with other stromal cell types (Fig. 1 a), even though intact reaggregate structures, providing optimal conditions for interaction, formed in all cases. Thus, both fetal mesenchyme and thymus-derived dendritic cells failed to support maturation, even though the latter express both MHC class I and II molecules (13), providing the potential for selection of both CD8+ and CD4+ cells, respectively. Similarly, other types of fetal epithelium (gut and salivary gland), even when induced to express MHC class II molecules by prior culture in IFN-γ, also failed to support thymocyte survival and maturation, indicating that positive selection had not taken place.

It is important to note that the lack of positive selection by cells other than thymic epithelium was not due to the inability of CD4+CD8+ TCR- cells to reach the CD4+CD8+ TCR+ stage (when selection can operate) in the absence of thymic support. Indeed, the majority of CD4+CD8+ TCR- cells (>95%) survived for the first 18 h of culture and acquired low levels of αβTCR expression, either when cultured in the complete absence of stromal cells (Fig. 2, c and d), or in the presence of IFN-γ-treated nontumoral stromal cells (Fig. 2, e and f). This latter observation also excludes the possibility that IFN-γ-treated stromal cells failed to sup-
**Figure 1.** Comparison of the abilities of various thymic and nonthymic stromal cells to support positive selection of CD4+ CD8+ thymocytes in vitro. Reaggregate lobes, consisting of purified CD4+CD8- TCR- thymocytes and individual stromal cell types, were harvested after 4 d in culture and analyzed by microscopy for total thymocyte content (a). (b and c) Flow cytometric analysis of thymocytes harvested from reaggregate cultures formed from purified MHC class II+ thymic epithelium. Note that TCR+, CD4+, and CD8+ cells have been generated from the TCR-CD4+CD8+ input. After 4 d, insufficient viable thymocytes for analysis were recovered from reaggregates formed from other stromal cells.

**Figure 2.** Purified CD4+CD8+TCR- thymocytes progress to a αβTCR+ stage in short-term culture independent of stromal cell support. Immunomagnetically selected CD4+CD8+TCR- thymocytes were analyzed by flow cytometry for their expression of CD4, CD8, and αβTCR either immediately after isolation (a and b), or after culture for 18 h, either in the complete absence of stromal cell support (c and d), or in the presence of IFN-γ-treated salivary epithelium (e and f). Gates defining the levels of αβTCR expression were set by comparison with adult thymocytes stained in parallel. Although no viable cells could be found in these cultures after 4 d, consistent with the finite life span of CD4+ CD8+ cells in the absence of positive selection, at 18 h the majority of cells (>95%) were viable, still CD4+CD8+ (c and e), and had progressed to a stage where most of the cells expressed low levels of αβTCR (d and f). Data shown are representative of three separate experiments.
Pathways. In conclusion, the most likely explanation of our findings is that only thymic epithelial cells can supply all the signals required for positive selection and the consequent maturation of T cells via other developmental signals. In the maturation of CD4+CD8+ thymocytes. Three separate experiments were performed with similar results. Note that in both cases, single positive CD4+ and CD8+ cells with upregulated levels of TCR have been generated from the input of untreated (a and b) or ECDI-treated thymic stromal cells (c and d), to demonstrate their ability to support positive selection (Fig. 3, c and d). Thus, it is likely that the additional signals that thymic epithelial cells provide for positive selection of thymocytes involve ligands on the cell surface rather than soluble products.

Overall, our results show that thymic epithelial cells are unique in their ability to provide positive selection signals for the maturation of CD4+CD8+ thymocytes into CD4+ or CD8+ T cells. Since thymic epithelial cell-specific peptide–MHC complexes do not appear to be required for positive selection (8, 9), this suggests that as well as TCR ligation, thymic epithelial cells provide additional signals necessary for positive selection. Such a requirement for dual signaling in positive selection parallels that seen in the activation of mature T cells, where both TCR ligation and costimulatory signals are required (17). However, despite some similarities between positive selection and activation (18), the additional signals involved are unlikely to be the same, since MHC class II+ thymic epithelial cells do not express the costimulatory ligand B7 and are unable to deliver functional costimulatory signals for the activation of mature T cells (13).

Finally, our results are in agreement with a recent study (19) where introduction of MHC class II-bearing, bone marrow–derived cells into the thymus of MHC class II–deficient mice failed to allow the positive selection of class II–restricted CD4+ cells. However, they differ from other studies (8, 9) where MHC class I–bearing cells introduced into the thymus of MHC class I–deficient mice by intrathymic injection or the construction of bone marrow chimeras did allow positive selection of class I–restricted CD8+ cells. Whereas this may reflect differences in the selection signals required for CD4+ and CD8+ cells, we did not observe selection of either cell type by individual stromal elements other than thymic epithelium. This suggests that the formation of both T cell types requires interaction with thymic epithelial cells. Thus, positive selection by MHC–peptide complexes on cells other than the thymic epithelium may only be possible when thymic epithelial cells are also present to provide the additional accessory signals required for maturation. Our current studies are aimed at defining the surface molecules on thymic epithelial cells that provide these accessory signals.

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Address correspondence to Dr. G. Anderson, Department of Anatomy, The University of Birmingham Medical School, Vincent Drive, Edgbaston, Birmingham B15 2TT, UK.

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