Specialized Ability of Thymic Epithelial Cells to Mediate Positive Selection Does Not Require Expression of the Steroidogenic Enzyme P450scc

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Thymic epithelial cells are uniquely efficient in mediating positive selection, suggesting that in addition to providing peptide/MHC complexes for TCR ligation, they may also provide additional support for this process. Recent studies have shown that although engagement of either the TCR or glucocorticoid (GC) receptors can individually induce apoptosis in thymocytes, together these signals are mutually antagonistic. This had led to the suggestion that local GC production by thymic epithelial cells, by opposing TCR signaling for apoptosis, provides the basis of the ability of these cells to mediate thymocyte positive selection. In this paper we have examined this possibility directly and shown that highly purified cortical epithelial cells, which have the functional ability to mediate positive selection in reaggregate cultures, do not express mRNA for the key steroidogenic enzyme P450scc. Thus we conclude that the ability of thymic epithelial cells to support positive selection does not rely on their ability to produce GC. However, we find that P450scc mRNA is up-regulated in thymocytes on the initiation of positive selection, raising the possibility that any local protective effect of steroid production is mediated at the level of thymocytes themselves. The Journal of Immunology, 1999, 163: 5781–5785.

Initial selection of the TCR repertoire occurs at the CD4\(^+\)CD8\(^-\) stage of thymocyte development when TCR-mediated interaction with MHC/peptide complexes on the thymic stroma lead to either positive or negative selection. A key element in determining which of these responses takes place is now known to be the overall avidity of TCR ligation. Thus, lower avidity interactions can induce positive selection, whereas high avidity interactions trigger negative selection (1, 2) through the induction of apoptosis (3). In addition, there is evidence that accessory signals or factors provided by specialized stromal cells are also crucial in determining the outcome of TCR-mediated selection (4). Thus, we have shown that cortical epithelial cells of the thymus are unique in their ability to mediate positive selection efficiently (5), whereas bone marrow-derived dendritic cells are known to be efficient mediators of negative selection (6).

In the case of negative selection by dendritic cells, there is some evidence that accessory signals can be provided by costimulatory molecules such as CD80/CD86, which are known to be important in the activation of mature T cells (7). However, the nature of the specialized support provided by thymic epithelial cells for positive selection is unknown. Interestingly, however, a recent suggestion is that glucocorticoid (GC)\(^1\) production by thymic epithelial cells is an important element in their ability to mediate positive selection (8–11). This is based on the observation that, whereas TCR signaling and GC treatment can independently induce apoptosis in T cell hybridomas (11) and developing thymocytes (8, 9), together these signals are mutually antagonistic. Thus, occupancy of the GC receptor at the same time as TCR ligation during thymocyte development could allow the activation of signaling pathways leading to positive selection while simultaneously suppressing pathways leading to negative selection (10). This suggestion has been further supported by reports of local GC production in the thymus and expression of the steroidogenic enzyme P450scc by a subset of radio-resistant thymic cells (8). However, the relationship of these cells to the stromal cells mediating selection events is unclear.

In this study, we have directly examined the possible role of GC production by thymic stromal cells in thymocyte selection by comparing expression of the mRNA that encodes the key steroidogenic enzyme P450scc in highly purified cortical epithelial cells with the functional ability of these cells to mediate positive selection. Because P450scc mRNA is found only in tissues with steroidogenic potential and because it is considered the determining factor in steroid production (12), analysis of its expression is a definitive marker for the potential for GC synthesis (8). We show that, although P450scc mRNA expression is readily detectable in whole thymus lobes, purified thymic epithelial cells that are efficient mediators of positive selection in reaggregate organ cultures (RTOC) do not express this enzyme. In contrast, expression of P450scc mRNA is detectable in thymocytes undergoing positive selection, as defined by expression of the activation marker CD69 (13). Thus, our data suggest that the ability to produce GC does not underlie the specialized ability of thymic epithelial cells to mediate positive selection. However, our results leave open the possibility that induction of endogenous steroid production in thymocytes in response to TCR ligation could play a role in regulating signals leading to positive selection.

Materials and Methods

Mice

BALB/c (H-2\(^b\)) mice were bred and maintained at the Biomedical Services Unit, University of Birmingham. Embryos were obtained from timed...
mats, with the day of detection of the vaginal plug being designated as day 0.

Abs and immunocoujugates

Anti-rat or anti-mouse IgG-coated Dynabeads (Dynal, Wirral, U.K.) were coated as appropriate with the following Abs: anti-CD3 (clone KT3; a kind gift from Dr. Julian Dyson, Medical Research Council Clinical Sciences Centre, London, U.K.); anti-CD8 (clone YTS169.4; Sera-Lab, Crawley Down, Sussex, U.K.); anti-CD45 (clone M1/9; American Type Culture Collection, Manassas, VA); and anti-IA^d (clone AMS-32.1; PharMingen, San Diego, CA). DNA-coupled anti-mouse IgG-coated beads (Collection beads, Dynal) were also coated with anti-IA^d for use in epithelial cell isolation. The following Abs were used for flow cytometry of isolated stromal cells and thymocytes: anti-CD8 FITC (clone 53-6.7), and anti-CD4 PE (clone GK1.5).

Cell separations

Preparation of thymocytes. Prepositive selection CD4^+ 8^+ thymocytes were prepared from newborn thymus cell suspensions by immunomagnetic depletion of CD3^+ cells, then by positive selection on CD8 as described previously (14, 15).

Isolation of thymic stromal cells. Thymus lobes from 15-day mouse embryos were cultured in 2-deoxyguanosine (2-dGuo) for 5–7 days to deplete lymphoid precursors and were trypsinized to form single cell suspensions as described previously (14, 15). Residual hemopoietic elements were immunomagnetically depleted with anti-CD45-coated beads. If required, further purification of MHC class II^+ thymic epithelial cells was obtained by positive selection using anti-IA^d bound to anti-mouse IgG-coated Dynabeads, or anti-IA^d bound to DNA-coupled anti-mouse IgG-coated Collection beads. Magnetically isolated positive cells were released from the beads either by brief exposure to pronase in the case of anti-IgG-coated beads as described previously (16), or for DNA-linked beads by the use of the release buffer according to the manufacturers instructions.

Formation of RTOC

Freshly prepared thymocytes and appropriate stromal cells were mixed together in 1.5-mL Eppendorf tubes at a ratio of 2:1 and pelleted by centrifugation. After removal of the supernatant, the cell pellet was carefully transferred using a micropipette to the surface of a 0.8-micron nuclear pore filter (Corning Costar, High Wycombe, U.K.) in organ culture. Intact thymus lobes reformed in these cultures within 1–2 h (14–16).

Flow cytometric analysis

Thymocytes were harvested from RTOCs by gentle mechanical disruption using fine knives. Thymocyte suspensions and/or stromal cell suspensions were analyzed following immunolabeling using a dual-laser Coulter Epics Elite machine (Coulter, Hialeah, FL) with forward and side scatter set to exclude nonviable cells (16).

RT-PCR

RT-PCR was conducted as described previously (17). Briefly, total RNA was extracted from ~5-10^3 cells using TRIzol (Life Technologies, Paisley, Scotland) according to the manufacturer’s instructions. To remove any contaminating genomic DNA, RNA samples were treated with RNase-free DNase I (Pharmacia Biotech, Uppsala, Sweden). Reverse transcription was conducted according to Montgomery and Dallman (18) using Moloney murine leukemia virus reverse transcriptase (Life Technologies) with first strand synthesis primed using oligo(dT). β-Actin (internal control for RT-PCR) was used as a housekeeping gene to obtain equivalent amounts of cDNA in each sample. cDNA (5 µl) was added to reaction mixtures (100 µl total volume) containing 1× GeneAmp PCR buffer, consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% (w/v) gelatin (Perkin-Elmer, Warrington, Cheshire, U.K.), 200 µM dNTPs (Pharmacia Biotech), 1 µM of forward and reverse primers, and 2.5 U AmpliTaq gold DNA polymerase (Perkin-Elmer). The MgCl₂ concentration for β-actin, P-450, and IL-7 oligonucleotides are as follows: β-actin: sense, 5′-GTACCCAACCTGGGACGACA-3′; anti-sense, 5′-TGCGCATCTTCCTGCTGAA-3′; P450sc: sense, 5′-CCTGTGGGACAGTATGCTGGC-3′; anti-sense, 5′-GGCAACACCCAGCGGCA-3′; IL-7: sense, 5′-ACTACACCCACCTCCCGCA-3′; anti-sense, 5′-TCTAGATGCTCTTTGAGG-3′.

Reactions were conducted in a Perkin-Elmer Thermal Cycler PTC-200 (MJ Research, Genetic Research Instrumentation, Dunmow, Essex, U.K.). The first step of the reaction involved activation of AmpliTaq gold DNA polymerase at 94°C for 12 min; then cycles of 94°C for 30 s, annealing of primers β-actin, IL-7, and P-450 at 50°C, 55°C, and 60°C, respectively, for 40 s; and extension of primed template at 72°C for 1 min. At various cycles, samples were cooled to 4°C before removing 10 µl aliquots, to allow for semiquantitation. PCR products were analyzed by ethidium bromide agarose gel electrophoresis and identified by fragment size.

Results and Discussion

Detection of P450sc mRNA in fetal thymus organ culture

Previous studies have shown the expression of the key steroidogenic enzyme P450sc in adult murine thymus in vivo (8–11). To examine expression in fetal thymus organ cultures, which support a full program of T cell development in vitro, thymus lobes were isolated at day 15 of gestation and placed in organ culture over a period of 7–8 days. During this time, these cultures support the generation of double positive CD4^+ 8^+ cells and their positively selected CD4^+ or CD8^+ progeny (19). Whole lobes harvested at this stage and analyzed by RT-PCR showed clear expression of P450sc when compared with β-actin-matched adult adrenal gland as a positive control (Fig. 1).

FIGURE 1. P450sc is expressed strongly in lymphoid but only weakly in epithelial-enriched alymphoid thymus organ cultures. Thymus lobes from day 15 gestation mouse embryos were explanted in organ culture either in the absence of 2-dGuo to generate lymphoid thymic lobes (Whole Thymus) or in the presence of the 1.35 mM 2-dGuo, which selectively depletes the lymphoid compartment (Whole Stroma). cDNA was synthesised from lobe extracts and semiquantitative RT-PCR analysis was performed for expression of the key steroidogenic enzyme P450sc, with levels of β-actin used to ensure equal cDNA loading between samples. As a positive control for P450sc expression, adult mouse BALB/c adrenal glands were used. Data shown are representative of three separate experiments.
whole thymus lobes depleted of lymphoid and dendritic cell precursors by treatment with 2-dG (20). Such lobes consist of a mixture of cortical MHC class II+ epithelial cells, medullary epithelial cells, mesenchymal fibroblasts, and macrophages (14). As shown in Fig. 1, low levels of P450scc mRNA expression were detected in these preparations; however, highly purified preparations of MHC class II+ epithelial cells isolated from dGuo-treated lobes (Fig. 2, a and b) showed no detectable levels of P450scc mRNA expression (Fig. 3). Importantly, when such preparation of purified epithelial cells were incorporated into RTOCs with purified preselection CD4+8+ thymocytes (Fig. 2c), they were able to support the maturation of these cells through positive selection resulting in the generation of single positive CD4+ and CD8+ cells (Fig. 2d).

Although these findings suggest that the capacity for local steroid production by thymic epithelial cells is not essential to their ability to mediate positive selection, analysis of purified epithelial cells from alymphoid lobes leaves open the possibility that steroid production by epithelial cells is only induced as a result of interaction with lymphoid cells. To explore this possibility, we prepared reaggregate cultures of purified CD4+8+ thymocytes and purified MHC class II+ epithelium. These cultures were maintained for 4 days to allow interactions supporting positive selection to proceed (see Fig. 2, c and d), then disaggregated, and class II+ epithelial cells were purified from these dispersions by immunomagnetic selection. RT-PCR analysis of epithelial cells reisolated from such cocultures with lymphoid cells also showed no expression of P450scc (Fig. 3), although these preparations still expressed detectable levels of IL-7, previously shown to be selectively expressed by MHC class II+ epithelial cells of the thymic stroma (17). Thus, expression of this molecule by epithelial cells is not induced by, or dependent upon, interaction with thymocytes. Taken together, our results show that highly purified MHC class II+ epithelial cells, capable of supporting positive selection, do not express the mRNA for the key steroidogenic enzyme P450, indicating that local steroid production by these cells is not essential to their function in mediating positive selection.

**FIGURE 2.** Purification of MHC class II+ thymic epithelial cells from whole thymic stroma correlates with their ability to mediate positive selection. 2-dGtreated thymus lobes were disaggregated with 0.25% trypsin in 0.02% EDTA and analyzed by flow cytometry for MHC class II expression both before (a) and after (b) immunomagnetic selection. Purified epithelial cells are routinely found to be >98% MHC class II+ upon reanalysis. Purified CD4+8+ TCR- thymocytes (c), when reaggregated for 5 days with purified epithelial cells shown in b, undergo positive selection and maturation into single positive CD4+CD8- and CD4+8+ cells (d). Data shown are representative of three separate experiments.

**FIGURE 3.** P450scc is not expressed in highly purified, positively selecting thymic epithelium, either before or after coculture with CD4+8+ thymocytes. Purified thymic epithelial cells from freshly trypsinized 2-dGuo-treated thymus lobes were either tested immediately for P450scc expression by RT-PCR (purified epithelium); or reaggregated with CD4+8+ thymocytes for 5 days, harvested from disaggregated reaggregate cultures, and then analyzed for P450scc expression (cocultured epithelium). The latter allows investigation of the possibility that P450scc expression in epithelial cells is dependent upon contact with thymocytes undergoing positive selection. As in Fig. 1, adrenal gland tissue was used as a positive control for P450scc expression, whereas expression of IL-7 in thymic preparations was used to ensure the integrity of cDNA isolated from these preparations and to underline its epithelial origin. In all cases, β-actin levels were used to monitor equal gel loadings. Data shown are representative of three separate experiments.
Correlation of P450scc expression with thymocyte subsets during positive selection

In view of the expression of P450scc in whole thymus lobes and its absence from MHC class II⁺ cortical epithelial cells, we next analyzed its expression in the lymphoid compartment. Thymocytes were purified on the basis of their expression of CD4, CD8, and CD69 to give a series of developmental subsets before and after the induction of positive selection. Within this series, CD4⁺8⁻69⁻ thymocytes represent preselection cells, whereas CD4⁺8⁺ cells that also express CD69 represent cells that have received initial signals for positive selection (21, 22). As shown in Fig. 4, preselection CD4⁺8⁻TCR²⁺ thymocytes do not express detectable levels of P450scc, but this is up-regulated on positive selection as shown by its detection in CD4⁺8⁺69⁺ cells and increased expression as these cells mature to the single positive stage. Interestingly, this expression is transient, as shown by the absence of P450scc mRNA expression in mature CD3⁺ T cells isolated from adult BALB/c lymph nodes (data not shown).

To obtain further direct evidence that P450scc up-regulation is associated with positive selection, we also compared expression in purified CD4⁺8⁺ cells (Fig. 2c) before incorporation into RTOCs, with the progeny of these cells recovered from reaggregate cultures where positive selection takes place (Fig. 2d). As shown in Fig. 5, although the input population of purified CD4⁺8⁺ cells lacks P450scc, expression is readily detectable in the lymphoid cells recovered from these cultures, which are predominantly newly generated CD4⁺8⁻ and CD4⁺8⁺ cells (Fig. 2d). Thus, our use of an in vitro system of positive selection confirms that up-regulation of P450scc expression is associated with the induction of positive selection in developing thymocytes.

FIGURE 4. Expression of P450scc mRNA in thymocytes correlates with the induction of positive selection. Thymocyte subsets representing preselection CD4⁺8⁻ TCR⁻ thymocytes (DP TCR⁻), CD4⁺8⁺69⁻ cells that have initiated positive selection (DP CD69⁺), and newly selected single positive cells (SP CD4⁺ CD69⁻) were purified from newborn thymus and analyzed by semiquantitative RT-PCR. Samples matched for β-actin show that P450scc is undetectable in preselection CD4⁺8⁻ TCR⁻ thymocytes, but that it appears in CD4⁺8⁺69⁻ thymocytes, with increased expression on continued maturation to the single positive CD4⁺ stage.

FIGURE 5. Induction of differentiation of CD4⁺8⁺ thymocytes into single positive cells in reaggregate cultures is accompanied by the up-regulation of P450scc expression. To investigate changes in P450scc expression during positive selection in vitro, CD4⁺8⁺ thymocytes (see Fig. 2c) were reaggregated with purified MHC class II⁺ thymic epithelial cells (see Fig. 2b) and cultured for 5 days. Thymocytes harvested from these cultures (see Fig. 2d) were subsequently analyzed for P450scc expression. Note that, in agreement with the data shown in Fig. 4, CD4⁺8⁺ (DP TCR⁻) cells used as reaggregate inputs lack expression of P450scc, whereas β-actin-matched thymocytes harvested from reaggregate cultures (reaggregate output), in which positive selection to the CD4⁺8⁻ and CD4⁺8⁺ stage takes place, have readily detectable levels of P450scc. Data shown are representative of three separate experiments.
Positive selection of developing thymocytes for progression from the CD4\(^+\)8\(^+\) double positive to the CD4\(^+\) or CD8\(^+\) single positive stage is now known to require low/medium avidity TCR-mediated interaction with MHC peptide ligands (1, 2). In addition, we have shown that expression of these ligands, specifically on cortical epithelial cells of the thymus, is essential for efficient positive selection (5). This suggests that these cells possess additional properties supporting or driving the positive selection process. Definition of these properties remains one of the key unresolved issues in thymus biology.

In this context, recent studies have demonstrated that GC, which normally induce apoptosis in cortical thymocytes, are able to antagonize TCR-mediated signaling, leading to apoptosis in these cells. This has led to the mutual antagonism hypothesis which proposes that occupancy of the thymocyte GC receptor during low/medium avidity TCR interactions allows positive rather than negative selection to occur, although high avidity signaling triggering negative selection is able to overcome this antagonism (8–11). Together with the demonstration of local GC production in the thymus, this has raised the possibility that GC production by thymic epithelial cells is the key to their specialized ability to mediate positive selection. In this paper, we have provided unequivocal evidence that thymic epithelial cells, able to mediate positive selection, do not express mRNA for the key steroidogenic enzyme P450scc. Hence, we are able to exclude the possibility that the ability of thymic epithelial cells to make GC underlies their capacity to mediate positive selection.

Although our results eliminate a role for GC of epithelial origin, they do not exclude a role for GC antagonism of TCR signaling in allowing positive selection to take place. Rather, our observation that P450scc mRNA is up-regulated in thymocytes undergoing TCR-induced selection raises the possibility that endogenous steroid production by thymocytes allows TCR signals in the appropriate avidity range to trigger positive selection without inducing apoptosis. Further support for this hypothesis would come from the demonstration of GC production by newly selected thymocytes in addition to its expression at the mRNA level.

Finally, with a role for GC production by thymic epithelial cells excluded, the unique properties that enable these cells to support positive selection are still to be defined. As one approach, we are carrying out a survey at both RNA and protein levels to identify genes that are differentially expressed in thymic epithelial cells compared with cells that lack the ability to mediate positive selection.

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**References**