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Block of T lymphocyte differentiation by activation of the cAMP-dependent signal transduction pathway

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Differentiation of T lymphocytes is a complex and finely tuned process. Here we show that treatment of mouse fetal thymus organ cultures with agents activating the cAMP-dependent signalling pathway results in the block of thymocyte differentiation. This is due to severe impairment of maturation beyond the CD4–/CD8– stage. In addition, rearrangements at the TCRα gene locus, but not at the TCRβ locus, are completely inhibited. The cAMP effect is reversible and is restricted to TCRαβ+ cells. cAMP acts both by triggering apoptosis and by inducing cell-cycle block in thymocytes. Thus, activation of the cAMP pathway provides a mechanism to modulate thymic function for hormones and ligands whose receptors are coupled to adenylate cyclase.

Keywords: cyclic AMP/lymphocyte development/signal transduction

Introduction

T lymphocyte differentiation involves a highly ordered series of events by which CD34+ stem cells incoming from the bone marrow become mature CD4+ or CD8+ T cells (Fowlkes and Pardoll, 1989; Spits, 1994). During their ontogenesis, T cells undergo selection processes which allow mature lymphocytes to express antigen receptors which recognize non-self peptides presented in the context of self-MHC class I and II molecules (Marrack and Kappler, 1987). The MHC co-receptors CD4 and CD8 are expressed simultaneously at the stage where the selection processes take place (Fowlkes and Pardoll, 1989). Gene targeting experiments have shown that the expression of a functional αβ T cell receptor (TCRαβ) during development is essential for progression of the differentiation programme. In fact, thymocytes from TCRα-null mice do not proceed beyond the CD4+/CD8+ double-positive (DP) stage, while transition of cells from the CD4–/CD8– double-negative (DN) stage to the DP stage is impaired by elimination of the TCRβ chain gene (Mombaerts *et al.*, 1992; Philpott *et al.*, 1992). However, little is known about the intracellular mechanisms that are involved in the regulation of this differentiation programme.

Here we show that activation of the cAMP signalling pathway modulates thymocyte differentiation. cAMP is produced upon stimulation of specific receptors coupled via G-proteins to adenylate cyclase and interacts with the protein kinase A (PKA) regulatory subunit, leading to the phosphorylation of proteins present both in the cytoplasm and the nucleus (Habener, 1990; Borrelli *et al.*, 1992; Lalli and Sassone-Corsi, 1994). The final result of signals delivered through the cAMP pathway varies according to the cell type; cAMP is usually mitogenic and a stimulator of hormone secretion in endocrine cells, while for cells of the immune system it generally delivers an 'off' signal for many differentiated functions, e.g. mast cell degranulation, interleukin (IL)-1 release by macrophages and IL-2-dependent T cell-cycle progression (K.W.Johnson *et al.*, 1988; Kammer, 1988). The molecular bases of the differential cAMP action are poorly understood.

Here we show that activation of the cAMP signalling pathway in the thymus can block differentiation at an early stage, both by triggering apoptosis and by blocking the thymocyte cell cycle. This causes a dramatic decrease in the number of DP thymocytes, which express very low levels of CD3. The block of the differentiation process involves selectively TCRαβ+ cells, while the TCRγδ+ lineage is not affected. Selective pharmacological stimulation of β-adrenoreceptors produces analogous effects, suggesting that thymocyte differentiation *in vivo* can be modulated by adrenergic inputs acting through the cAMP pathway.

Results

Stimulation of the cAMP-dependent signalling pathway in the thymus blocks lymphocyte differentiation

We established fetal thymus organ cultures (FTOC) derived from day 14 mouse embryos. Dibutyryl-cAMP (diBut-cAMP), a cAMP cell-permeable analogue, was added to a subset of these cultures. Thymocytes were phenotyped 5 days later. We found that diBut-cAMP treatment produced a dose-dependent decrease in cell recovery and of the number of DP, but not of DN thymocytes (Figure 1A and C; Table I). In addition, the cell number of CD4+ single positives (SP) in treated FTOC was significantly reduced, while CD8+ SP cell number was not. This is consistent with the notion that CD4+ SP cells are mostly derived from the DP population, while a certain proportion of intrathymic CD8+ cells does not belong to the TCRαβ+ lineage (Fowlkes and Pardoll, 1989; see below). Accordingly, the average cell size in cAMP-treated FTOC is larger (Figure 1B), due to the depletion of DP cells, which are mostly smaller than DN precursors.

To confirm that the differentiation block was due to activation of the cAMP pathway and to exclude non-specific toxic effects of diBut-cAMP, other compounds

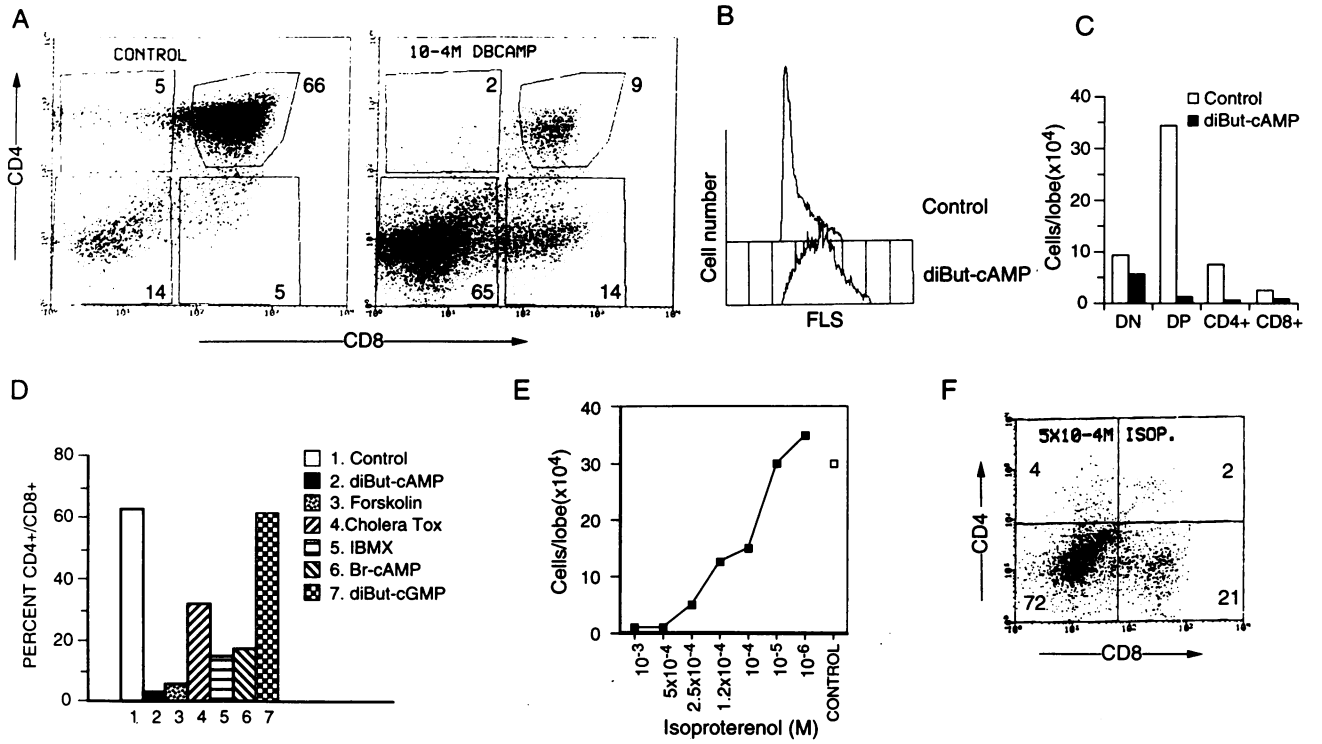

Fig. 1. Agents that activate the cAMP-dependent signalling pathway block thymocyte differentiation. (**A**) Phenotype of cells from day 5 FTOC, untreated (left) and treated (right) with 10^{-4} M diBut-cAMP. Thymocyte suspensions were stained with anti-CD4 and anti-CD8 monoclonal antibodies. Cell percentages in windows are shown for a typical experiment. (**B**) Forward light scatter (FLS) profile of control (top) and 10^{-4} M diBut-cAMP-treated (bottom) FTOC. The ratio between the mean FLS of treated and control FTOC was 1.22 ± 0.01 (mean ± SEM of eight experiments). (**C**) Mean cell recovery per lobe (eight experiments) for CD4+, DP, DN and CD8+ thymocytes from control (white histograms) and 10^{-4} M diBut-cAMP-treated (black histograms) FTOC. In each case, SEM did not exceed 15% of the mean; DP and CD4+ cell numbers were significantly different ($P<0.01$; Mann–Whitney U test) between control and diBut-cAMP-treated FTOC. (**D**) Percentages of CD4+/CD8+ DP cells in day 5 untreated control and 10^{-4} M diBut-cAMP-, 20 μg/ml forskolin-, 200 μg/ml cholera toxin-, 100 μg/ml IBMX-, 200 μM 8-bromo-cAMP- and 10^{-4} M diBut-cGMP-treated FTOC. (**E**) Cell recovery per lobe in FTOC treated for 5 days with various doses of the β-adrenoreceptor agonist isoproterenol. Black squares: isoproterenol-treated FTOCs. White square: day 5 FTOC cultured in control conditions. This experiment was performed three times; variability was <10%. (**F**) CD4+/CD8+ phenotype of cells recovered from 5×10^{-4} M isoproterenol-treated FTOC. Numbers indicate the percentage of cells in each quadrant.

Table I. diBut-cAMP treatment of FTOC causes a dose-dependent reduction of cell yield

diBut-cAMP	DN	DP	CD4+	CD8+	Total
0	3.8×10^4	3.5×10^5	4.7×10^4	1.5×10^4	4.5×10^5
10^{-5} M	6.7×10^4	1×10^5	1×10^4	1.6×10^4	1.9×10^5
10^{-4} M	4.1×10^4	2.4×10^4	5×10^3	9.7×10^3	7.9×10^4
10^{-3} M	2.4×10^4	2×10^3	1.4×10^3	2×10^3	2.9×10^4

FTOC were set up from day 14 mouse embryos on Nucleopore polycarbonate filters in DMEM supplemented with 10% FCS and penicillin–streptomycin. Various doses of diBut-cAMP were added to a subset of FTOC, and thymic lobes were dissociated by syringing after 5 days from the onset of culture. Cells were counted with a haemocytometer and phenotyped. Data for the various populations are expressed as number of cells per thymic lobe in culture.

known to stimulate the cAMP pathway were used. Forskolin and cholera toxin (which activate adenylate cyclase), IBMX (3-isobutyl-1-methyl-xanthine, an inhibitor of cell phosphodiesterases) and 8-bromo-cAMP (a slowly hydrolysable cAMP analogue) all produced the same effect (Figure 1D). Moreover, diBut-cGMP, which regulates a different set of cellular kinases and does not interact with the PKA regulatory subunit, had no effect (Figure 1D).

Physiological significance

Lymphocytes express a variety of membrane receptors which are coupled to adenylate cyclase (E.W.Johnson *et al.*, 1988; Dobner *et al.*, 1992; Birkenbach *et al.*, 1993; Emrich *et al.*, 1993; Kaplan *et al.*, 1993; Kouba *et al.*, 1993; Santambrogio *et al.*, 1993). The thymus is richly innervated by adrenergic terminations (Kendall and Al-Shawaf, 1991; Bellinger *et al.*, 1992; Leposavic *et al.*, 1992), and thymocytes express significant levels of β-adrenoreceptors (Singh *et al.*, 1979); importantly, the thymic response to adrenergic stimuli is modulated during immune processes, being depressed upon an antigenic challenge and increased thereafter (Morale *et al.*, 1992).

Selective stimulation of β-adrenergic receptors in FTOC by isoproterenol produces a dose-dependent reduction in cell recovery (Figure 1E). FTOC treatment with iso-

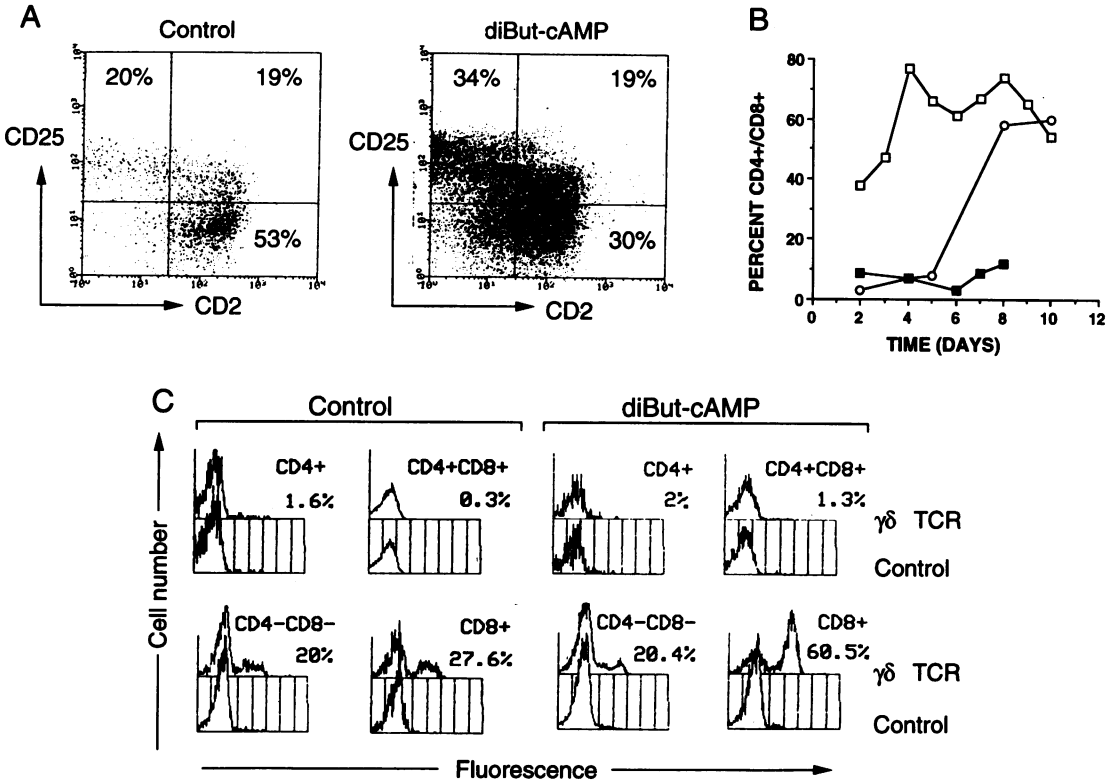

Fig. 2. Phenotype of cAMP-treated FTOC. (**A**) CD25/CD2 staining of CD4–/CD8– DN cells from (left) day 5 untreated and (right) 10^{-4} M diBut-cAMP-treated FTOC. (**B**) Percentages of CD4+/CD8+ DP cells at different times in control FTOC (white squares), 10^{-4} M diBut-cAMP-treated FTOC (black squares) and FTOC treated with diBut-cAMP for 5 days and then transferred to fresh medium (white circles). (**C**) TCRγδ+ cells in day 5 control and 10^{-4} M diBut-cAMP-treated FTOC. Triple staining was performed with the anti-TCR δ chain antibody (Ab) 3A10, anti-CD4 and anti-CD8. The fluorescence profiles and percentages of TCRγδ+ cells in the CD4+, DP, DN and CD8+ subpopulations are shown. The mean number of cells in the control cultures was 2×10^4 cells/lobe while it was 2.1×10^4 cells/lobe in cAMP-treated cultures. As negative control the staining with non-specific hamster Ab is also shown.

proterenol mimics the diBut-cAMP effect, since DN cells are preserved while more mature cells selectively disappear (compare Figure 1A and F). The specificity of the stimulated receptor is crucial, since treatment with corticotrophin-releasing factor and adrenocorticotrophin, whose receptors are also present on lymphocytes (E.W.Johnson *et al.*, 1988; Clarke and Bost, 1989; Grigoriadis *et al.*, 1993), has no effect (data not shown). The effect of isoproterenol is antagonized by equimolar concentrations of the specific β-adrenoreceptor antagonist propranolol (not shown).

Thus, stimulation of a specific membrane receptor that activates adenylate cyclase can reproduce the effect elicited by diBut-cAMP and other agents increasing the intracellular cAMP concentration.

An early stage in differentiation is impaired by cAMP treatment

We wished to identify the differentiation stage within the DN cellular population where the cAMP-directed block occurs. During thymocyte differentiation DN cells first express CD25. CD25+ cells then become DP after CD25 down-modulation and acquisition of CD2 (Ceredig, 1990; Rodewald *et al.*, 1993). Thus, we analysed the phenotype of DN cells by studying CD25 and CD2 expression in cAMP-treated and control FTOC. cAMP only marginally influenced the transition of DN thymocytes from the CD25+/CD2– stage to the CD25–/CD2+ stage (Figure 2A), thus determining the stage of block at the boundary between late (CD2+) DN and early DP.

The cAMP-induced block is reversible

The effect of cAMP on FTOC is reversible. Day 5 FTOCs were transferred from medium with diBut-cAMP to fresh medium, and the reappearance of DP cells was monitored. Five days after the transfer (equivalent to day 10 of culture), the DP cell population had returned to the levels of the untreated FTOC control (Figure 2B). These data exclude possible genetic imprintings by cAMP on precursor cells, and correlate with the reversibility of its effects on TCRα gene rearrangements (see below).

cAMP treatment of FTOC does not affect the TCRγδ+ lineage

Our results indicate that activation of the cAMP pathway inhibits the differentiation of TCRαβ+ lineage cells (Figure 1). Differentiation of TCRγδ+ cells does not involve the transition from a DN to a DP stage; most TCRγδ+ thymocytes express neither CD4 nor CD8, whilst some are CD8+ SP (Fowlkes and Pardoll, 1989; Fisher and Ceredig, 1991). Strikingly, the differentiation of TCRγδ+ cells was not influenced by diBut-cAMP (Figure 2C). The phenotype and cell recovery of TCRγδ+ cells in treated FTOC was similar to controls. Their percentage reached

~20% of all cells in cAMP-treated FTOC (forming most of the remaining CD8+ SP cells), compared with 4% in control cultures.

Activation of the cAMP pathway inhibits TCRα chain gene rearrangements and subverts thymic architecture

To assess whether the few residual DP cells present after cAMP treatment are phenotypically normal, we analysed their CD3 expression. This population expresses low levels of CD3 compared with untreated FTOC DP cells (Figure 3A). This phenotype is reminiscent of the low, but detectable CD3 expression in DP thymocytes from FTOC of TCRα –/– mice (Philpott *et al.*, 1992; Figure 3A). DP cells from cAMP-treated FTOC are also stained very weakly with an anti-TCRβ mouse antibody (not shown), while analysis of TCRα was impaired by the lack of suitable antibodies to detect its surface expression. These findings suggest that DP thymocytes from cAMP-treated FTOC lack a mature TCRαβ–CD3 complex. To confirm this hypothesis, we analysed the extent of TCRβ and α chain gene rearrangements in isolated DN and DP cells from untreated and cAMP-treated day 5 FTOC.

TCRβ chain gene rearrangements were not detectable in embryonic day 14 thymic lobes (not shown). Strikingly, while β chain gene rearrangements in FTOC occurred normally in the presence of diBut-cAMP (as detected by amplification of rearrangements of the $V_{\beta3}$, $V_{\beta6}$, $V_{\beta8}$ and $V_{\beta17}$ gene families to the $J_{\beta2.5}$ region and to C_β), α chain gene rearrangements ($V_{\alpha5H}$, $V_{\alpha F3}$ and $V_{\alpha2C}$ to $J_{\alpha TT11}$) were completely inhibited (Figure 5B).

The integrity of the lymphocyte differentiation programme is essential for the development of thymus architecture (van Ewijk *et al.*, 1994). Thus, we wished to investigate whether the striking effect on thymic differentiation could correlate with morphological changes in the tissue upon treatment. Histological analysis of cAMP-treated FTOCs shows a complete subversion of thymic architecture. In untreated FTOCs there exists a sharp demarcation of thymic cortex from the medulla (Figure 3C). Conversely, cAMP-treated FTOCs (Figure 3C) are atrophic and lack a well-developed medulla and cortico-medullary boundary. In addition, cystic structures, which are small in untreated FTOCs, become enlarged.

Restoration of DP thymocyte differentiation, by transferring day 5 cAMP-treated cultures to fresh medium for another 5 days, correlated with the appearance of TCRα gene rearrangements (Figure 4B). In addition, analysis of CD3 and TCRβ chain surface expression from recovered cultures showed a distribution typical of early FTOC (Figure 4A). Taken together, these results constitute strong evidence that mature TCRαβ are not expressed on the surface of DP thymocytes from cAMP-treated FTOC.

cAMP does not alter p56^{lck} activity in treated FTOC

Overexpression of a dominant-negative form of the CD3-associated p56^{lck} kinase blocks thymocyte differentiation and inhibits TCRα gene rearrangements (Levin *et al.*, 1993). This finding suggests that signals mediated through p56^{lck} regulate recombination at the TCRα gene locus. To determine whether cAMP treatment modulates p56^{lck} activity, cell extracts from control and cAMP-treated FTOC were assayed. There was no decrease in p56^{lck} levels and autophosphorylation activity measured on immunoprecipitates upon cAMP treatment (Figure 4C).

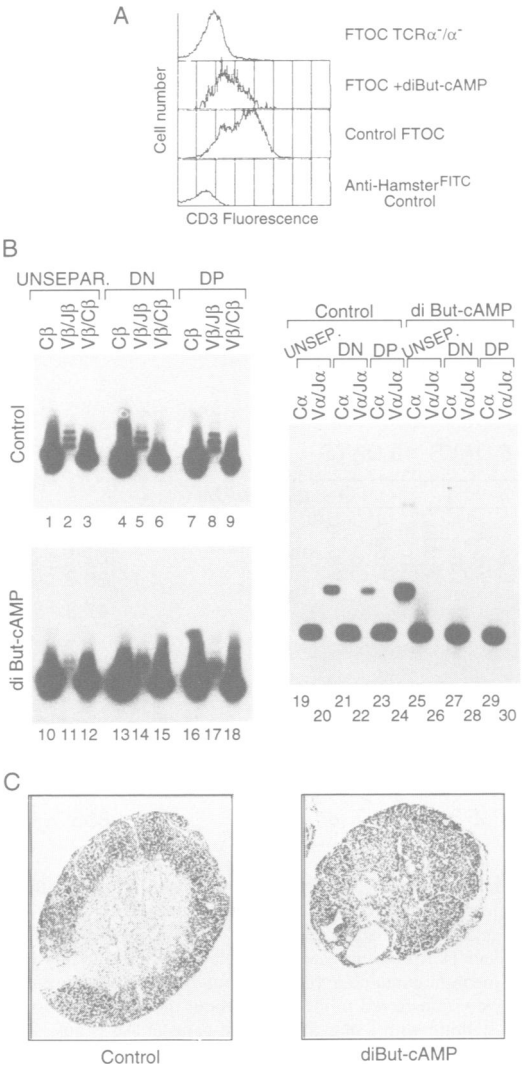

Fig. 3. cAMP treatment inhibits TCRα gene rearrangements and induces drastic changes in thymic architecture. (**A**) CD3 expression on CD4+/CD8+ DP cells of (from top to bottom): day 5 FTOC from TCRα$^-$/α$^-$ mouse embryos; day 5 FTOC treated with 10^{-4} M diBut-cAMP; day 5 untreated FTOC. Fluorescence of an anti-hamster antibody control is also shown. (**B**) PCR analysis of TCRβ chain (left; lanes 1–18) and TCRα chain (right; lanes 19–30) gene rearrangements. Lanes 1–3 and 19–20: unseparated thymocytes from control FTOC. Lanes 4–6 and 21–22: sorted DN from control FTOC. Lanes 7–9 and 23–24: sorted DP from control FTOC. Lanes 10–12 and 25–26: unseparated thymocytes from diBut-cAMP-treated FTOC. Lanes 13–15 and 27–28: sorted DN from diBut-cAMP-treated FTOC. Lanes 16–18 and 29–30: sorted DP from diBut-cAMP-treated FTOC. Lanes 1, 4, 7, 10, 13 and 16: C_β; lanes 19, 21, 23, 25, 27 and 29: C_α (internal controls). For TCRβ chain gene rearrangement, results are shown for rearrangement of the $V_{\beta3}$ variable region gene family to $J_{\beta2.5}$ (lanes 2, 5, 8, 11, 14 and 17) and C_β (lanes 3, 6, 9, 12, 15 and 18). Equivalent results were obtained using 5′ primers specific for the $V_{\beta6}$, $V_{\beta8}$ and $V_{\beta17}$ variable region gene families (not shown). For TCRα chain gene rearrangement, PCR was performed using as 5′ primers a mixture of oligonucleotides specific for the $V_{\alpha5H}$, $V_{\alpha F3}$ and $V_{\alpha2C}$ variable region gene families (lanes 20, 22, 24, 26, 28 and 30) and as 3′ primer an oligonucleotide specific for $J_{\alpha TT11}$. (**C**) Histological sections of (left) day 5 control and (right) 10^{-4} M diBut-cAMP-treated FTOC. Thymic lobes were fixed in 4% paraformaldehyde, paraffin-embedded and hematoxylin/eosin stained. ×120 magnification.

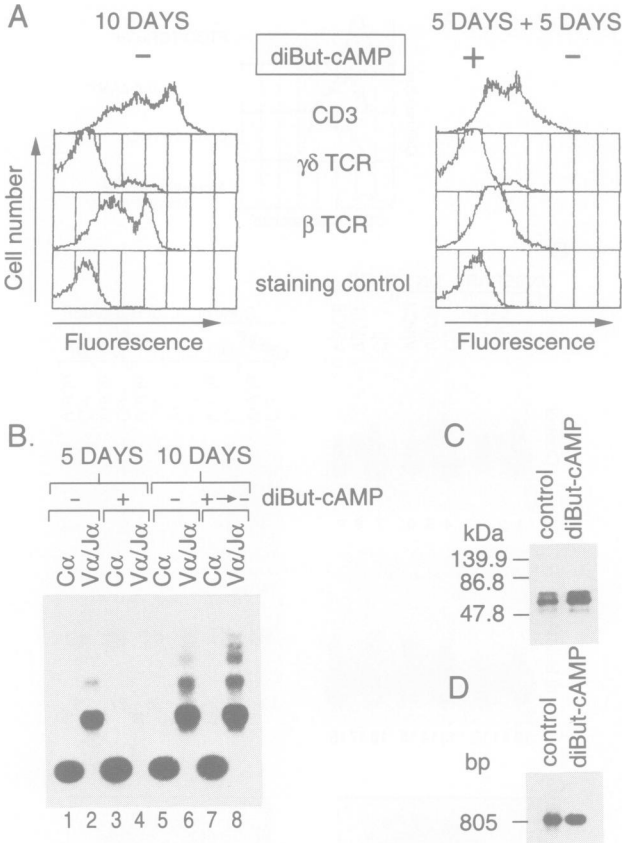

Fig. 4. Inhibition of TCRα gene rearrangements by cAMP treatment is reversible and cannot be accounted for by diminished $p56^{lck}$ kinase activity and TEA transcript expression. (**A**) Expression of CD3, TCRγδ and TCRβ by untreated day 10 FTOC (left), and by FTOC kept in medium containing 10^{-4} M diBut-cAMP for 5 days and successively transferred to medium without the compound for 5 days (right). Staining with a negative control hamster Ab is also shown. Lack of a $CD3^{bright}$, $TCR\beta^{bright}$ population in day 5 cAMP-treated FTOC thymocytes is evident, indicating their developmental retardation compared with FTOC cultured without cAMP from the beginning. (**B**) PCR analysis of TCRα gene rearrangements from day 5 (lanes 1–4) and day 10 (lanes 5–8) FTOCs. Lanes 1, 3, 5 and 7: C_α. Lanes 2, 4, 6 and 8: $V_{\alpha 5H}$, $V_{\alpha F3}$ and $V_{\alpha 2C}$ rearrangements to $J_{\alpha TT11}$. Lanes 1, 2, 5 and 6: FTOCs cultured without diBut-cAMP. Lanes 3 and 4: FTOCs cultured with diBut-cAMP for 5 days. Lanes 7 and 8: FTOCs cultured with diBut-cAMP for 5 days, then transferred to medium without the compound for another 5 days. (**C**) $p56^{lck}$ activity in control and diBut-cAMP-treated FTOC. The kinase was immunoprecipitated using a rabbit antiserum raised against a synthetic peptide corresponding to the N-terminus of human $p56^{lck}$ (UBI, Lake Placid, NY). Kinase assay was performed as described (Hurley and Sefton, 1989; Veillette *et al.*, 1989; Molina *et al.*, 1992) on immunoprecipitates. (**D**) TEA transcript expression in control and diBut-cAMP-treated FTOC. RT-PCR was performed on RNA extracted from FTOCs and TEA cDNA amplified as described (Shimizu *et al.*, 1993), using primers situated in the TEA exon and in the C_α second exon. PCR products were detected using an oligonucleotide probe corresponding to a sequence in the C_α first exon.

Increased cAMP levels do not affect germline transcription from the TCRα locus

In man, transcription from the T early α (TEA) genetic element located between C_δ and J_α precedes rearrangement at the TCRα gene locus. This event appears to be a decisive step in the recombination mechanism (de Villartay *et al.*, 1987). TEA transcript is also found in mouse thymus (Shimizu *et al.*, 1993). We were interested to know whether the cAMP-dependent inhibition of TCRα gene rearrangements could be associated with a transcriptional inhibition from the TEA element, thus providing a mechanistic explanation for the phenomenon. Surprisingly, cAMP-treated FTOC express approximately the same amount of TEA transcript as untreated FTOC (Figure 4D).

Table II. diBut-cAMP treatment of FTOC causes thymocyte apoptosis and cell-cycle block

		72 h		5 days	
		Control	diBut-cAMP	Control	diBut-cAMP
R1	M1	2.9	55.3	2	23.3
	M2	3.2	0.4	0.7	0.5
R2	M1	0.1	2.3	0	1.4
	M2	2.6	3	0.9	10.4
R3	M1	0	0.5	0	0.1
	M2	36.9	22.6	35.7	13.9

Thymocyte suspensions were recovered from diBut-cAMP-treated and untreated 72 h and 5 day FTOC. Cells were fixed in 70% ethanol, washed, RNase treated and stained with 50 μg/ml ethidium bromide. 30 000 events were collected on a FACScan flow cytometer, after gating out debris and aggregates by forward light scatter/red fluorescence signals. As shown in Figure 5A, the forward light scatter/90° light scatter graph was divided into three regions: R1 includes dead cells, R2 includes small live cells and R3 includes large live cells. The percentages of hypodiploid (M1; corresponding to apoptotic cells) and hyperdiploid (M2; corresponding to S/G_2–M cells) are reported for each of the three regions.

cAMP treatment of FTOC causes thymocyte apoptosis and cell-cycle block

To determine the mechanism underlying the decrease in thymocyte number following cAMP treatment, we analysed thymocyte DNA content in control and diBut-cAMP-treated FTOC at 3 days and 5 days after culture onset. After 72 h, almost all of the dead cells (R1 in Figure 5a) had a diploid DNA content in control FTOC (Table II), while 55.3% of R1 cells had a hypodiploid DNA content (M1 in DNA histograms in Figure 5a) in cAMP-treated FTOC. This is a characteristic sign of apoptosis (Nicoletti *et al.*, 1991). No major difference in cell-cycle profile between control and cAMP-treated FTOC was found for small live cells (R2 in Figure 5a). Conversely, the percentage of cells in the S/G_2–M phases of the cell cycle (M2 in DNA histograms in Figure 5a) was greatly enriched in control FTOC large live thymocytes (R3 in Figure 5a), compared with cAMP-treated FTOC large thymocytes (Table II). Thus, cAMP treatment causes a relative cell-cycle arrest in G_1. The DNA profiles were essentially the same at day 5, the only major difference being the large reduction in the hypodiploid peak of the R1 population in cAMP-treated FTOC, compared with day 3 (Figure 5a and Table II). The reduction of S/G_2–M cells in cAMP-treated FTOC was even larger at day 5 than at day 3 (Figure 5a and Table II), indicating that, at this time, the effect of cAMP on thymocyte differentiation is mainly due to cell-cycle block. Most of the remaining S/G_2–M cells in cAMP-treated FTOC are TCRγδ+ cells (not shown), which are actively proliferating in mouse fetal thymus and FTOC (Fisher and Ceredig, 1991), and whose number is not modified by cAMP treatment (see above). Thus, the causes of the reduced thymocyte yield in cAMP-treated FTOC are represented by both the triggering of apoptosis and the block of cell proliferation.

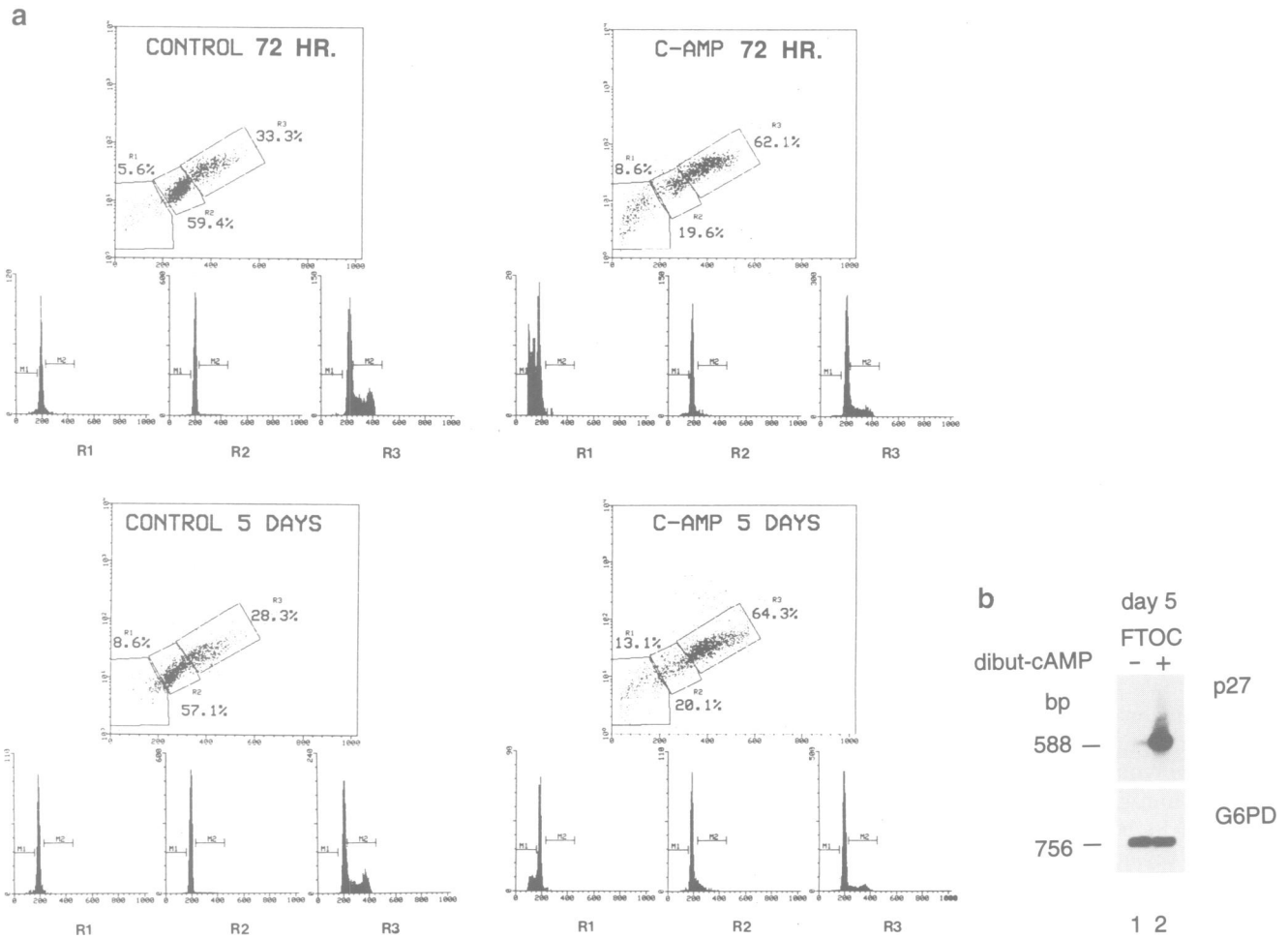

Fig. 5. DiBut-cAMP treatment of FTOC causes thymocyte apoptosis and cell-cycle block. (**a**) Cell-cycle analysis of FTOC thymocytes. Thymocyte suspensions were prepared from diBut-cAMP-treated and untreated FTOC kept in culture for 72 h (upper panels) and 5 days (lower panels). Cells were fixed in 70% ethanol, washed with PBS, treated with RNase A (20 μg/ml at 4°C for 30 min) and stained with 50 μg/ml ethidium bromide. DNA content was analysed with a FACScan flow cytometer, collecting 30 000 events after gating out debris and aggregates by forward light scatter/red fluorescence signals. The forward light scatter/90°C light scatter profile is plotted for each sample. Three regions were drawn on the light scatter dot plot: R1, which corresponds to dead cells, R2, comprising small live cells and R3, which includes large live cells. The percentage of cells within R3 is increased in diBut-cAMP-treated FTOC, compared with control, due to the larger average size of cells in the diBut-cAMP-treated cultures, as shown in Figure 1B. The DNA profile of the cells included in each region is shown. On each DNA histogram, two areas are indicated: M1 corresponds to cells with a hypodiploid DNA content (which is characteristic of apoptosis), and M2 includes cells in the S and G_2–M phases of the cell cycle. The percentages of cells with a hypodiploid and a hyperdiploid DNA content for each sample are reported in Table II. (**b**) DiBut-cAMP treatment induces the expression of the cdk4 cell cycle kinase inhibitor $p27^{Kip1}$ in FTOC. RNA was extracted from diBut-cAMP-treated and control day 5 FTOC by the guanidinium thiocyanate method. RNA was reverse-transcribed and PCR was performed (30 cycles) with two pairs of primers, which amplify, respectively, a 588 bp fragment of the $p27^{Kip1}$ mRNA (top panel) and a 756 bp fragment of G6PD mRNA (bottom panel). Amplified products were run on a 1.6% agarose gel, transferred to a nylon membrane and hybridized with internal oligos. Lane 1: untreated day 5 FTOC. Lane 2: diBut-cAMP-treated day 5 FTOC.

cAMP-triggered cell-cycle block is associated with induction of the cyclin-dependent kinase 4 inhibitor $p27^{Kip1}$

cAMP can antagonize colony-stimulating factor (CSF)-1 mitogenic effect on macrophages and produce their arrest in G_1 by inducing overexpression of the cyclin-dependent kinase 4 inhibitor $p27^{Kip1}$ (Kato *et al.*, 1994). Cell-cycle block represents one important consequence of cAMP treatment of FTOC. We tested whether an increased $p27^{Kip1}$ expression can account for this phenomenon in thymic lobes. While $p27^{Kip1}$ expression is very low in day 5 control FTOC (Figure 5b, lane 1), it is overexpressed in cAMP-treated FTOC (Figure 5b, lane 2). Expression of the housekeeping glucose-6-phosphate dehydrogenase (G6PD) gene was not affected by cAMP (Figure 5b).

Discussion

Signal transduction pathways in development

Involvement of signal transduction pathways modulated by membrane receptors in the control of cell differentiation is a feature extremely conserved during evolution. The *ras*–MAPK pathway has been shown to be required for photoreceptor differentiation in *Drosophila* (Dickson and Hafen, 1994), vulval cell differentiation in *Caenorhabditis elegans* (Eisenmann and Kim, 1994) and positive selection in thymocytes (Alberola-Ila *et al.*, 1995; Swan *et al.*, 1995). The cAMP-dependent signal transduction pathway also plays a pivotal role in *Dictyostelium* and *Drosophila* development (Simon *et al.*, 1992; Lane and Kalderon, 1993). In addition, recent evidence highlights the role of the

PKA pathway in the transduction of the signal generated by the *Drosophila* morphogen hedgehog (Blair, 1995).

We have shown that treatment of FTOC with compounds that increase intracellular cAMP levels inhibits thymocyte differentiation, blocking their maturation at a stage preceding the generation of DP cells. Isoproterenol, which selectively activates β-adrenergic receptors, also produces the same effect. The thymus is richly innervated by adrenergic fibres, which are especially abundant in the cortex (Kendall and Al-Shawaf, 1991; Bellinger *et al.*, 1992). Thymocytes express functional β-adrenergic receptors (Singh *et al.*, 1979), which are more abundant on DN cells (Rossi, 1987). Biphasic modulation of these receptors has been shown during the development of the immune response, suggesting that they can play an important role in the regulation of immune activation and subsequent suppression (Morale *et al.*, 1992). In addition, lymphocytes express several types of other G-protein-coupled membrane receptors, which are able either to activate or repress adenylate cyclase activity (Singh *et al.*, 1979; E.W.Johnson *et al.*, 1988; Dobner *et al.*, 1992; Birkenbach *et al.*, 1993; Emrich *et al.*, 1993; Kaplan *et al.*, 1993; Kouba *et al.*, 1993; Santambrogio *et al.*, 1993).

As shown here, cAMP stimulus blocks thymocyte differentiation prior to the DP stage. The regulation of the size of this population is critical to determine the number of mature lymphocytes released from the thymus. In fact, in TCR transgenic mice, where all thymocytes have the potential to be positively selected, the release of cells from the thymus is not markedly increased (Shortman and Scollay, 1994). Indeed, in these mice, there is an increased rate of DP to SP transition, but this is compensated by a reduction of the DP pool (Kelly *et al.*, 1993).

cAMP interferes with pre-T cell receptor signalling

The few DP thymocytes present in cAMP-treated FTOC have a distinct phenotype; they express low levels of CD3 and cannot express a functional TCRαβ, since rearrangements at the TCRα gene locus are completely blocked. The cellular composition of the thymus in TCRβ-null mice is strikingly similar to that of cAMP-treated FTOC (Mombaerts *et al.*, 1992; Malissen *et al.*, 1993). The TCRβ chain is expressed on immature DN thymocytes in association with a 33 kDa glycoprotein (pre-TCRα) and some components of the CD3 complex (Groettrup *et al.*, 1993; Saint-Ruf *et al.*, 1994) constituting an analogue of the TCR on immature thymocytes. The pre-T cell receptor delivers an intracellular signal which promotes cellular differentiation (Groettrup and von Boehmer, 1993; Mallick *et al.*, 1993). In fact, crossing a TCRβ transgene with rearrangement-deficient mice results in a dramatic increase in thymocyte number (Scott *et al.*, 1989; Mombaerts *et al.*, 1992; Shinkai *et al.*, 1993). Moreover, thymocyte number in pre-TCRα-deficient animals is reduced to $<10\%$ of that in littermate controls (Fehling *et al.*, 1995). This suggests that the pre-T cell receptor also delivers a proliferative signal and correlates with data showing that a larger proportion of late stage DN thymocytes expressing TCRβ chain is cycling, compared with their CD25+ precursors (Howe and MacDonald, 1987). In addition, it is known that the tyrosine kinase $p56^{lck}$ functionally couples to the TCRβ chain on immature thymocytes (Anderson *et al.*, 1992, 1993). Overexpression of a $p56^{lck}$ dominant-negative transgene blocked thymocyte differentiation in a dosage-dependent fashion (Levin *et al.*, 1993). Interestingly, in the lineage with the highest expression of the inactive kinase, TCRα gene rearrangements, but not TCRβ, were also completely inhibited, paralleling our findings in cAMP-treated FTOC. cAMP treatment, however, does not decrease $p56^{lck}$ kinase activity (Figure 4C). Our results suggest that signals activating the cAMP pathway can interfere at a downstream level with signals delivered through the pre-T cell receptor–CD3–$p56^{lck}$ functional complex, inhibiting rearrangements at the TCRα gene locus and blocking thymocyte differentiation. Therefore, treatment of FTOC with cAMP corresponds to functional ablation of the TCRβ chain, producing a phenotype analogous to TCRβ –/– mice. TCRα gene rearrangements, however, were shown to be present in TCRβ –/– mice (Mombaerts *et al.*, 1992), albeit at a reduced level. This may be explained by the possible activation to some extent of the pre-T cell receptor even in the absence of the β chain. This notion is supported by the observation that antibodies against the CD3 ε chain can restore DP cell generation in FTOC from TCRβ –/– mice (Levelt *et al.*, 1993). In this scenario, TCRα gene rearrangements can be totally inhibited only by interference with signals incoming from the pre-T cell receptor, as in the case of treatment of FTOC with diBut-cAMP or overexpression of a high-copy $p56^{lck}$ dominant-negative transgene.

Effect on cell cycle and apoptosis

Activation of the cAMP pathway causes inhibition of thymocyte differentiation by increasing cell death by apoptosis and by producing cell-cycle block. Our data clearly show that most dead thymocytes in cAMP-treated FTOC have a hypodiploid DNA content, which is typical of apoptosis (Nicoletti *et al.*, 1991). Considering that the number of DN thymocytes is not decreased significantly in cAMP-treated FTOC, cAMP-triggered apoptosis is likely to affect the more mature thymocytes selectively. Conversely, cAMP causes a cell-cycle block in large DN cells, which are actively cycling in control FTOC. Interestingly, rat sympathectomy results in a rise of the number of proliferating cells in the thymus cortex (Kendall and Al-Shawaf, 1991), indicating that adrenergic stimuli function tonically *in vivo* to depress thymocyte proliferation. The remaining cycling cells in cAMP-treated FTOC are mostly TCRγδ+ thymocytes, whose generation is not impaired under these conditions. The induction of the cyclin-dependent kinase 4 inhibitor $p27^{Kip1}$ (Kato *et al.*, 1994) in cAMP-treated FTOC may provide the explanation for the cAMP effect on the DN cell cycle, in a system where a direct link exists between cell proliferation and differentiation.

Regulation of gene rearrangements

We have also shown that cAMP treatment of FTOC does not modify expression of the TEA genetic element, located between the C_δ and the J_α locus. Transcription through this region appears to provide a local chromatin configuration accessible to the recombinase for cells belonging to the TCRαβ+ lineage. In mouse thymus, expression of TEA region transcripts during development parallels TCRδ and precedes TCRα expression (Shimizu *et al.*, 1993). The

demonstration that cAMP can inhibit TCRα gene rearrangements without modifying TEA expression constitutes an important finding, since it shows that transcription through this region is probably a necessary step, but not a sufficient condition, to produce TCRα gene rearrangements.

Lineage-specific effect

The number of CD4+ and CD8+ SP cells is greatly reduced upon cAMP treatment of FTOCs. While CD4+ cells are likely to be constituted mainly by early precursors (Wu *et al.*, 1991), the CD8+ population is composed of TCRγδ+ thymocytes and cells on the way to become DPs (MacDonald *et al.*, 1988). In fact, the absence of TCRαβ on the few remaining DP cells prevents their capacity to differentiate further and be positively selected (Ashton-Rickardt *et al.*, 1994; Hogquist *et al.*, 1994a,b), causing the striking reduction in CD4+ and TCRαβ+ CD8+ SP cells.

cAMP treatment of FTOC provokes a complete subversion of the thymic architecture, causing the disappearance of the compartmentalization into cortex and medulla. A similar absence of cortico-medullary demarcation is also present in SCID mice. The defect can be corrected by crossing these mice with double TCRαβ transgenic mice, but not by crossing with TCRβ transgenics (van Ewijk *et al.*, 1994). This finding shows the importance of thymocytes expressing the complete TCRαβ for the development of the thymic stroma, and parallels our results with cAMP-treated FTOC, where these cells are absent.

While the stimulation of the cAMP pathway completely blocks the generation of TCRαβ+ thymocytes, the TCRγδ+ lineage is not affected. In fact, TCRγδ+ cells constitute ~20% of the remaining cells in cAMP-treated FTOC, due to the massive depletion of DP and CD4+ SP cells. This may be explained by the fact that cAMP treatment blocks the thymocyte differentiation programme at a stage (the transition between late DN and DP cells) subsequent to the point where some precursors are already committed to the γδ lineage. Indeed, cAMP treatment does not impair the transition of DN thymocytes from the CD25+/CD2– to the CD25–/CD2+ stage (Figure 2A). Accordingly, it has been shown that the more immature CD44–/CD25+ DN population is the last subset able to repopulate the γδ component of lymphocyte-depleted FTOC (Godfrey *et al.*, 1993; Godfrey and Zlotnik, 1993). Others have found that CD44–/CD25–/CD4^{low}/CD8^{low} thymocytes are still capable of producing γδ T cells (Petrie *et al.*, 1992). Additional analysis suggests that the last possible stage of γδ T cell differentiation is the CD8+ immature thymocyte (Fisher and Ceredig, 1991).

Interestingly, the ablation of the pre-T cell receptor α gene selectively interferes with the development of the TCRαβ+, but not the TCRγδ+ lineage (Fehling *et al.*, 1995). This finding further reinforces our hypothesis that the stimulation of the cAMP-dependent signal transduction pathway selectively interferes with pre-T cell receptor signalling. It is possible that cAMP action on thymocyte development is, at least in part, mediated through an effect on thymic stromal cells, causing the disappearance of a ligand or cytokine selectively needed for TCRαβ+, but not TCRγδ+, cell development. Further studies are needed to address this point.

Studies involving overexpression of a p56^{lck} dominant-negative transgene (Levin *et al.*, 1993), crossing of TCRβ chain transgenes with recombination-deficient mice (Scott *et al.*, 1989; Mombaerts *et al.*, 1992; Shinkai *et al.*, 1993; Godfrey *et al.*, 1994), *in vivo* deletion of the TCRβ and pre-TCRα genes (Mombaerts *et al.*, 1992; Fehling *et al.*, 1995) and effect of cAMP on thymocyte differentiation (this paper) identify a precise stage in the thymocyte differentiation process. This stage corresponds to the very boundary between DN and DP, the point where the decision about the cell lineage (TCRαβ+ versus TCRγδ+) is made. At this stage, TCRα gene rearrangements are activated, and differentiation to the DP stage is triggered by signalling through the TCRβ chain/pre-T cell receptor. Progression through this stage can be counteracted by overexpression of a p56^{lck} dominant-negative transgene (Levin *et al.*, 1993) or by treatment of FTOC with cAMP (our study), which impair both TCRα gene rearrangements and generation of DP. It is interesting to note that these two phenomena, while taking place at the same stage in development, are distinct, and there is no apparent causal relationship between them. In fact, some DP cells, although abnormal, can be generated even in cAMP-treated FTOC, where TCRα gene rearrangements are completely inhibited. While it is clear that signals generated through the pre-T cell receptor are necessary for maturation to the DP stage (Mallick *et al.*, 1993; Fehling *et al.*, 1995), here we show that signals activating the cAMP pathway may counteract the effect of the former. Cyclic AMP, thus, in addition to its action on mature cells, represents a negative modulator for T lymphocytes also during their ontogeny.

Materials and methods

Fetal thymus organ cultures

Thymic lobes were removed from (C57BL/6×SJL) F2 day 14 mouse embryos and placed on Nucleopore polycarbonate filters (0.8 μm pore size), floating on 5 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 100 U/ml penicillin–100 μg/ml streptomycin, in 60 mm Petri dishes, as described (Ceredig, 1986). DiBut-cAMP, forskolin, cholera toxin, IBMX, 8-bromo-cAMP, isoproterenol and diBut-cGMP (all obtained from Sigma, St. Louis, MO) were added from the beginning of the culture. Thymic lobes were kept in culture for 5 days before phenotypic analysis and DNA or RNA extraction.

Flow cytometry

Cell suspensions were prepared by passing thymic lobes through hypodermic needles of decreasing size. Immunofluorescence staining and flow cytometry were performed as described (Ivanov *et al.*, 1993), utilizing the monoclonal antibodies 500A2 (anti-CD3), H57.597 (anti-TCRβ chain), 3A10 (anti-TCRδ chain), PC61 (anti-CD25), 12-15 (anti-CD2), G.K-1.5 (anti-CD4) and 53.6.7 (anti-CD8) (Becton Dickinson, Mountain View, CA). For cell-cycle analysis, cell suspensions obtained from FTOC were fixed in 70% ethanol at 4°C for 30 min, washed twice in phosphate-buffered saline (PBS), treated with 20 μg/ml RNase A at 4°C for 30 min and stained with 50 μg/ml ethidium bromide. Cells were analysed with a FACScan flow cytometer (Becton Dickinson), collecting 30 000 events per sample. Cell debris and aggregates were gated out by forward light scatter/red fluorescence signals. Data analysis was performed with the Lysis II software.

PCR

Genomic DNA was extracted from unseparated cells and from sorted DN and DP cells from control and cAMP-treated FTOC as described (Keller and Manak, 1989). Fifty ng of DNA per sample were analysed for TCRβ chain rearrangements by PCR as described (D'Adamio *et al.*, 1992), using primers 5′-GTCCTGTGGCGCTGCAGTGGTGAAAGG-GCA-3′ ($V_{\beta 3}$), 5′-CCTGATTGGTCAGGAAGGGC-3′ ($V_{\beta 6}$), 5′-TCC-

CTGATGGGTACAAGGCC-3′ ($V_{\beta 8}$), 5′-GTCCTGTGGCGCTGCAG-GCTCTTTATGTTGCT-3′ ($V_{\beta 17}$), 5′-TAACACGAGGAGCCGAGTGC-3′ ($J_{\beta 2.5}$), 5′-GCAAACAAACAAAAGGCTACC-3′ and 5′-CCACTT-GTCCTCCTCTGAAA-3′ (C_β). TCR α chain rearrangements were analysed by PCR as described (Levin *et al.*, 1993). Amplification of C_α DNA was performed by 5′ primer 5′-CAGAACCCAGAACCTG-CTGTG-3′ and 3′ primer 5′-GGCCCCATTGCTCTTGGAATC-3′, which amplify a 180 bp sequence in the C^α first exon. PCR products were run in 1× TBE on a 1.6% agarose gel, blotted on Hybond N+ nylon membrane and hybridized with internal 5′-AGAGACCCTCAGGCG-GCTGCTC-3′ (C_β), 5′-CTGGCCCAAAGTACTGGGTG-3′ (J_β), 5′-CGGCACATTGATTTGGGAGTC-3′ (C_α) and $J_{\alpha TT11}$ probes (Levin *et al.*, 1993).

For TEA transcript detection, RT-PCR was performed using 1 μg of RNA extracted from day 5 untreated or cAMP-treated FTOC (Chomczynski and Sacchi, 1987), as described (Kawasaki, 1990). For cDNA amplification, TEA exon 5′ primer and C_α CII exon 3′ primer were used, as described (Shimizu *et al.*, 1993). The Southern blot of PCR products was hybridized with the end-labelled C_α first exon probe.

For $p27^{Kip1}$ and G6PD transcript analysis, RNA was reverse-transcribed and cDNA amplified using the primers TCAAACGTGAG-AGTGTCTAACGGGAGC and CGTCTGGCGTCGAAGGCCGGGCT-TCTT ($p27^{Kip1}$) and the primers ATCTACCGCATTGACCACTACCTG and CCCACAGAAGACATCCAGGATGAG (G6PD). The internal oligos used for hybridization were GCGCAAGTGGAATTTCGACT ($p27^{Kip1}$) and TTCATCCTGCGCTGTGGCAAAGCTCTGAATGAGC-GCAAAGCTGAA (G6PD).

Immune complex kinase assay

Untreated and cAMP-treated thymic lobes were dissociated by needle aspiration. Immune complex $p56^{lck}$ kinase activity assay was performed as previously described (Hurley and Sefton, 1989; Veillette *et al.*, 1989; Molina *et al.*, 1992). Briefly, cells were lysed in ice-cold TNE buffer (50 mM Tris pH 8, 1% Nonidet P-40, 2 mM EDTA pH 8), supplemented with 200 μM sodium orthovanadate, 100 mM sodium fluoride and the protease inhibitors leupeptin and aprotinin (20 μg/ml each). After centrifugation, $p56^{lck}$ was immunoprecipitated from 100 μg of protein of each cell lysate using a rabbit antiserum raised against a synthetic peptide corresponding to amino acids 22–51 of human $p56^{lck}$ (UBI, Lake Placid, NY). Immunoprecipitates were washed twice in washing buffer (50 mM Tris pH 7.2, 150 mM NaCl) and once in kinase buffer (40 mM PIPES pH 7.1, 10 mM $MnCl_2$). They were then resuspended in 25 μl of kinase buffer containing 1 μM [γ-^{32}P]ATP (3000 Ci/mmol). After 2 min at 25°C, kinase reactions were stopped by addition of 1 ml of TNE buffer. Immunoprecipitates were then washed three times with TNE buffer, resuspended and boiled in Laemmli buffer, and run on a 10% SDS–PAGE gel.

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