In vitro differentiation of a CD4/CD8 double-positive equivalent thymocyte subset in adult Xenopus

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Abstract

The thymus is the major site of selection and differentiation of T cells in mammals and birds. To begin to study the evolution of thymocyte differentiation, we have developed, in the frog Xenopus, an in vitro system that takes advantage of cortical thymocyte antigen (CTX), a recently discovered T cell antigen whose expression is restricted to Xenopus cortical thymocytes. Upon transient stimulation with suboptimal mitogenic concentrations of the phorbol ester phorbol myristate acetate (PMA) plus ionomycin, Xenopus thymocytes are induced to differentiate into cycling T lymphoblasts that actively synthesize and express high levels of surface MHC class I and class II molecules. This appearance of T lymphoblasts correlates with a rapid down-regulation of both surface CTX protein and CTX mRNA. A thymocyte subset with an immature phenotype (CTX+CD8-, class II+ or class IIlow and class I-) was characterized by depleting class II+ cells or by panning with anti-CTX mAb. This immature CTX+ thymocyte subset displays a limited proliferative capacity compared to total, class II+ or to CTX- thymocytes, and can be induced, by PMA/ionomycin, to differentiate into more mature T lymphoblasts expressing surface class II and class I molecules. These results provide the first in vitro evidence in an ectothermic vertebrate of a conserved intrathymic pathway of thymocyte differentiation. In addition, our data reveal that CTX can serve as a differentiation surface marker of a population of immature thymocytes that appears to be the equivalent of the mammalian CD4/CD8 double-positive subset.

Introduction

In the mammalian thymus, positive and negative selection of immature T cells in the thymus occurs at the CD4/CD8 double-positive (DP) stage during or just after the last cell cycle of thymocyte expansion (1–3). Antigen-driven selection depends on the interaction of the newly formed CD3–TCRαβ complex with a peptide–MHC complex and on consecutive signal transduction events. It has been proposed (4–7) that a high-avidity interaction between the TCR and MHC-peptide complex results in apoptosis (negative selection), whereas lower avidity interactions induce thymic lymphocytes to differentiate further into single-positive CD4+CD8- or CD4-CD8+ thymocytes (positive selection). Recent in vitro studies with normal as well as with Rag2- or TCR α chain-deficient thymocytes indicate that a combination of moderate protein kinase C (PKC) activation and calcium elevation constitutes a biochemical signal that induces DP cells to differentiate into CD4+ T cells (8,9) and CD8+ T cells (10).

Although it has long been appreciated that the thymus of teleost fish, reptiles and amphibians clearly plays an essential role in the development of immunocompetence (11), little is known about thymocyte differentiation in these ectothermic vertebrates. This issue has become particularly important owing the rapid progress in the cloning of both MHC and TCR genes in variety of ectothermic species including Xenopus (12–15), axolotl (16–18), and teleostean (19) and cartilaginous fish (20–22). In view of this remarkable conservation of genes critical to the adaptive immune system, it is now imperative to obtain more direct evidence concerning the differentiation of T cell subsets and selection of the T cell repertoire in ectothermic vertebrates. Such studies have been hampered (i) by a lack of antibody reagents to identify T cells at various stages of their differentiation and (ii) by the lack of an in vitro system with which to reliably and effectively assay differentiation. To some extent, this first obstacle has been
resolved for the amphibian, Xenopus where mAb that recognize CD8 and MHC class I and II molecules are now available. Although mAb that identify Xenopus CD4 or TCR do not yet exist, mAb are available that recognize a Xenopus cortical thymocyte-specific cell surface molecule known as cortical thymocyte antigen (CTX). In the present study, CTX provided another marker of thymocyte differentiation since its expression is restricted to MHC class I cortical thymocytes, a cell population that may correspond to DP immature mammalian (23). CTX was first identified in Xenopus (23,24); subsequently, a homolog, called ChT1, was reported in chickens (25,26). In Xenopus, CTX displays structural features of a homodimeric cell surface receptor that is independent of the TCR–CD3 complex (24). Although the function of CTX is unclear, it has been implicated in the control of the cell cycle (27) of thymic tumor cell lines. ChT1 is also thought to be involved in T cell differentiation (25) and its expression by recent thymus emigrants (26) further suggests an important role of this new family of surface molecules in T cell development.

Until the present study, development of a reliable in vitro system with which to study thymocyte differentiation seemed problematic since Xenopus thymocytes (unlike splenocytes) respond poorly to mitogens like phytohemagglutinin or conavalin A (28–30). Although high concentrations of phorbol myristate acetate (PMA) do induce thymocyte proliferation, the cells rapidly undergo extensive apoptosis (31). Although high concentrations of phorbol myristate acetate (PMA) do induce thymocyte proliferation, the cells rapidly undergo extensive apoptosis (31). The consistency with which differentiation of mammalian thymocytes can be induced by a combination of a low concentration of PMA plus ionomycin (9) prompted us to adapt this approach can be induced by transient exposure to PMA/ionomycin at doses that are minimally mitogenic. Furthermore, these data establish CTX as a differentiation marker of an immature thymocyte subset that appears equivalent to the CD4/CD8 double-positive subset of mammals.

**Methods**

**Thymocyte cultures**

Thymuses from five to six 6-month-old outbred frogs were pooled, dissociated and cultured in 24-well flat-bottom plates (2×10⁶/1.5 ml/well) in Iscove’s DMEM basal medium diluted to amphibian osmolarity and supplemented with 5% FBS and 20% supernatant from the A6 kidney fibroblast cell line (ATCC: CCL 102) as detailed elsewhere for lymphoid tumor cell lines (32). PMA at a final concentration from 0.1 ng/ml to 200 ng/ml and ionomycin at a final concentration from 200 ng/ml to 1.6 μg/ml, were added directly to culture wells from 10 times concentrated stock solution of each made up in culture medium. Proliferation assays were performed by culturing 2-5×10⁴ thymocytes/well (in 200 μl volume) with or without PMA/ionomycin for the indicated periods of time. Cells were pulsed for the last 20 h with 1 μCi/well [³H]thymidine (Amersham) and harvested with a 96-well harvester (Betaplate, Wallac, Turku, Finland); thymidine uptake was determined by scintillation spectrometry.

Depletion of adult class II⁺ thymocytes was performed using magnetic beads (Dynal, Oslo, Norway) according to the manufacturer’s instructions. Briefly, 2 ml of M-450 beads coated with sheep anti-mouse IgG (Dynal) was incubated overnight with 50 ml of Xenopus anti-class II mAb (AM20) (10⁵) hybridoma supernatants (33). Thymocytes (2×10⁵) were washed twice with amphibian PBS and then incubated at 4°C for 1 h with washed conjugated magnetic beads in 10 ml final volume of medium. After magnetic separation, cells were washed and the efficacy of the depletion was determined by flow cytometry. Consistently, >90% of class II⁺ cells were removed; cell death, as measured by Trypan dye exclusion, was <5%. Class II⁺ cells were recovered from magnetic beads by an overnight incubation in medium and several washes.

Adult CTX⁺ thymocytes were positively selected by panning on six-well plates coated with anti-CTX mAb X71 supernatants as described elsewhere (27). Thymocytes (2×10⁸ in a volume of 2 ml/well) were incubated at 26°C for 1 h; non-adherent cells were then removed by gentle agitation and flushing. Adherent cells, recovered after an overnight incubation at 26°C, were washed twice and cultured with or without PMA/ionomycin. This protocol enriched a starting population of thymocytes that contained 50–60% CTX⁺ cells to one that was >95% CTX⁺; cell death was always <5%.

**Flow cytometry and immunocytochemistry**

Samples of 10⁵ cells were stained with undiluted hybridoma supernatants followed by fluorescein-labeled goat anti-mouse Ig; 10,000 cellular events were analyzed by flow cytometry on a Elite cytometer (27). Dead cells, detected by propidium iodide (10 μg/ml), as well as forward and side scatter, were gated out. The mAb directed against CTX [X71, 1S9.2 (23,24)], CD8 [AM22 (34)], MHC class I antigens [TB17 (35)], class II antigens [AM20 (33)], CD5 [2B1 (36)] and pan-T cells [XT1 (37)] were used. Gates were set to exclude 95–98% of the cells that stained with non-specific isotype-matched antibodies.

**Biosynthetic labeling and immunoprecipitation**

Thymocytes, cultured for 4 days in Iscove’s-derived medium with or without PMA/ionomycin, were harvested, washed twice in amphibian PBS and then incubated for 45 min at a density of 2.5×10⁵/ml in pre-warmed methionine/cysteine-free RPMI medium diluted to amphibian molarity. After centrifugation, the cells were resuspended (5×10⁵/ml) in the same medium supplemented with 5% dialyzed FBS and 20% A6 supernatant, and containing 2 μCi/ml of [³⁵S]methionine/cysteine (Pro-Mix # SJQ.0079). After 20 h incubation, cells were harvested, washed twice and lysed (1–5×10⁸/ml) in RIPA buffer. Radiolabeled cell lysates (10⁶ c.p.m./ml corresponding to ~10⁶ cells) were pre-cleared for 1 h at 4°C with 30 μl/ml of protein G; 100 μl of this pre-cleared lysate was incubated overnight at 4°C with 100 μl of mAb supernatants and 30 μl Protein G. Immunoprecipitates were separated on 10% SDS-PAGE gels under reducing or non-reducing conditions.

**RT-PCR**

Cytoplasmic RNA was prepared from 5×10⁷ total thymocytes that were either untreated or treated overnight with PMA/ionomycin according to the vanadyl–ribonucleoside complex method (38). First-strand cDNA synthesis was performed
**Results**

**Effects of PMA and ionomycin on adult thymocytes in vitro**

Two subsets appear after stimulation. PMA and/or ionomycin were titrated separately or in combination, in cultures of total thymocytes (Fig. 1). Ionomycin (at all concentrations tested) did not induce any marked proliferation. Higher concentrations (1–2 µg/ml) of PMA stimulated some transient proliferation but also induced significant cell death as reported previously by Hsuet al. (31). The greatest proliferation was obtained with a combination of PMA (10–100 ng/ml) plus ionomycin (400 ng/ml). Proliferation, already significant by 24 h of culture (Fig. 1), markedly increased after 2–3 days of culture and persisted for at least 6 days. After 2 weeks, thymidine incorporation rapidly decreased and cells began dying even after PMA/ionomycin was removed by washing and the cells were replated in just fresh medium. Thymidine uptake was markedly reduced by using a lower dose of ionomycin (200 ng/ml, Fig. 1). At all doses used, PMA/ionomycin also induced cell death (Table 1).

Proliferation of thymocytes correlated with the appearance of a subset of large lymphoblastoid cells which were often found in aggregates. On Giemsa-stained cytospin preparations, this lymphoblast subset differed from a second subset of small thymocytes by their greater amount of cytoplasm and their mitotic activity, as well as by their larger size (data not shown). This lymphoblast subset could also be identified as an actively cycling population and gated from small resting thymocytes by flow cytometry (Fig. 2B). It corresponded to 30–40% during the first day of culture and to >50% of the total viable lymphocyte population after 4 days. In the absence of PMA/ionomycin, this cell subset never exceeded 5%.

**CTX, CD8 and MHC surface expression are modulated in the lymphoblast subset.** The general pattern of expression of different Xenopus markers used in this study is outlined in Table 2. Compared with thymocytes initially put into culture, the two cell subpopulations present in PMA/ionomycin 4 day cultures displayed a very different cell surface phenotype (Fig. 2A and C). The small thymocytes resembled the thymocytes cultured for 4 days in medium alone (see Fig. 5A and B). They retained their surface expression of CTX and CD8 molecules but they had become class II⁺. These cells were also CD5⁺, XT1⁺ and weakly class I⁺ (class I⁺low). Consistently, 5–10% of this subset of small thymocytes expressed more CD8 than freshly harvested thymocytes (Fig. 2C).

The lymphoblast subset present in PMA/ionomycin 4 day cultures also displayed a distinct surface phenotype compared with that of the starting population of thymocytes. Surface CTX was undetectable, CD8 was markedly decreased, and both class I and class II surface expression was sharply increased. These lymphoblasts belong to the T cell lineage because they expressed the XT1 marker which is found on >90% of total thymocytes and peripheral T cells (37) (see Fig. 5B) and did not stain with anti-Xenopus IgM mAb (data not shown).
Table 1. Live cell number, percent dead cells, and percent T lymphoblasts in cultures of unselected and selected thymocyte subsets before and after 4 days of culture with/without 10 ng/ml PMA plus 400 ng/ml ionomycin (or 0.2 ng/ml PMA plus 200 ng/ml ionomycin)*

<table>
<thead>
<tr>
<th></th>
<th>At start of culture</th>
<th>After 1 day of culture</th>
<th>After 4 days of culture</th>
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<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>+ PMA/ionomycin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>live cell no. (× 10⁶)</td>
<td>2.0</td>
<td>2.15 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>percent cell death</td>
<td>4.0 ± 1.0</td>
<td>9.1 ± 1.2</td>
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<tr>
<td></td>
<td>percent T lymphoblasts</td>
<td>2.0 ± 1.2</td>
<td>4.0 ± 1.7</td>
</tr>
<tr>
<td>Thymocytes class II-depleted</td>
<td>live cell no. (× 10⁶)</td>
<td>2.0</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>percent cell death</td>
<td>6.0, 6.2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>percent T lymphoblasts</td>
<td>2.0, 3.0</td>
<td>ND</td>
</tr>
<tr>
<td>Class II⁺ thymocytes</td>
<td>live cell no. (× 10⁶)</td>
<td>2.0</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>percent cell death</td>
<td>6.0, 5.8</td>
<td>ND</td>
</tr>
<tr>
<td>CTX⁺ enriched thymocytes</td>
<td>live cell no. (× 10⁶)</td>
<td>2.0</td>
<td>0.72, 0.65</td>
</tr>
<tr>
<td></td>
<td>percent cell death</td>
<td>5.8, 6.0</td>
<td>25, 29</td>
</tr>
<tr>
<td></td>
<td>percent T lymphoblasts</td>
<td>1.5, 2.0</td>
<td>ND</td>
</tr>
<tr>
<td>CTX⁻ thymocytes</td>
<td>live cell no. (× 10⁶)</td>
<td>2.0</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>percent cell death</td>
<td>5.2, 6.0</td>
<td>ND</td>
</tr>
<tr>
<td>Total (unselected) thymocytes + low dose of PMA/ionomycin*</td>
<td>live cell no. (× 10⁶)</td>
<td>2.0</td>
<td>1.8, 1.7</td>
</tr>
<tr>
<td></td>
<td>percent cell death</td>
<td>4.2, 3.6</td>
<td>5.2, 4.9</td>
</tr>
<tr>
<td></td>
<td>percent T lymphoblasts</td>
<td>2.0, 2.5</td>
<td>5.1, 3.2</td>
</tr>
</tbody>
</table>

Data from total thymocytes are the means ± SD of four different experiments.
* Determined by Trypan blue dye exclusion.
ND, not done

Table 2. Expression pattern of the markers currently available in Xenopus

<table>
<thead>
<tr>
<th>Xenopus markers (mAb) in adult</th>
<th>Expression pattern in the thymus</th>
<th>Expression pattern in the periphery</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>CD8 (AM22)</td>
<td>Mainly thymic cortex (80% of total thymocytes)</td>
<td>T cells (~20% of splenocyte)</td>
<td>34</td>
</tr>
<tr>
<td>CTX (XT71)</td>
<td>Thymic cortex, 50–70% total thymocytes</td>
<td>No consistent expression in periphery</td>
<td>23, 40</td>
</tr>
<tr>
<td>XT1 (XT1)</td>
<td>Thymic cortex and medulla</td>
<td>Most, but not all, T cells</td>
<td>37</td>
</tr>
<tr>
<td>MHC class I (TB17)</td>
<td>Mainly thymic medulla, weak staining</td>
<td>Ubiquitous, expressed by all lymphopoietic lineages</td>
<td>30</td>
</tr>
<tr>
<td>MHC class II (AM20)</td>
<td>Mainly thymic medulla, bright staining</td>
<td>Both B and T cells (99% of splenocytes)</td>
<td>33, 34</td>
</tr>
</tbody>
</table>

No mAb specific for CD4 and TCR have been described so far.

The observed changes reflect differentiation rather than activation. Immunoprecipitation of biosynthetically labeled unseparated thymocytes that had been cultured for 4 days with 10 ng/ml PMA plus 400 ng/ml ionomycin indicated that many cells were actively synthesizing class II molecules (at least 10 times more than untreated thymocytes) and, to a lesser extent, class I molecules. However, with respect to CD8 and CTX molecules, they were virtually inactive biosynthetically (Fig. 3A).

Given the lack of appropriate molecular probes with which to further analyze the complexities of Xenopus CD8 expression (e.g., the gene has not been cloned), we focused our subsequent analysis on CTX as a marker of differentiation. RT-PCR was performed to more directly assess the effect of PMA/ionomycin on CTX gene regulation. In contrast to untreated thymocytes, CTX cDNA could not be detected by amplification of cytoplasmic RNA reverse-transcribed from thymocyte culture treated overnight either with 10 plus 400 ng/ml or 0.2 plus 200 ng/ml of PMA and ionomycin respectively (Fig. 3B).

Since large amounts of Ef-1α cDNA could be amplified both from treated and untreated cDNA samples, all these cells were metabolically active.

To determine further whether the phenotypic changes induced in vitro were due to activation (reversible) or differentiation (irreversible), thymocytes were only transiently exposed (16 h) to PMA/ionomycin. Cell phenotype was analyzed before (day 0), directly after washing (day 1) or after thymocytes had been put back in culture without PMA/ionomycin (day 2,
PMA/ionomycin-treated cells were actively incorporating thymidine (Fig. 4B, top right graph), suggesting that the lymphoblast population was actively renewed. Interestingly, a fraction of T lymphoblasts still expressed surface CTX at a low level after 16 h of treatment with PMA/ionomycin (day 1; Fig. 4B bottom right graph), indicating that PMA/ionomycin treatment does not only eliminate CTX^+ thymocytes, but also down-regulates CTX expression in a fraction of them.

The high level of class II surface expression by the whole T lymphoblast population remained stable from the first day of culture. A rather constant fraction of T lymphoblasts was also CD8^+, whereas in these culture conditions, surface expression of class I became significant only at day 2 (1 day after being replated in absence of PMA/ionomycin).

The fact that T lymphoblasts are generated in vitro following transient exposure to PMA/ionomycin, as well as the fact that this treatment induces rapid down-regulation of CTX mRNA and up-regulation of MHC class I and class II proteins, strongly suggest that a differentiation process, rather than an activation, process has been induced.

**Effect of PMA/ionomycin on selected subsets of thymocytes**

Freshly harvested thymocytes were enriched for immature cells either by depleting mature class II^+ cells or by positively selecting CTX^+ cells. Depletion of class I^+ thymocytes was not attempted because class I surface expression on thymocytes from young adults is very weak. As seen in Fig. 2(A), ~90% of the total thymocytes from 4- to 6-month-old adults are class II^+, although the intensity with which they stained varied over 3 log. After magnetic bead depletion of class II^+ cells, only a residual 10% of thymocytes with a low class II-specific signal was detectable (Fig. 5A). These class II^+low cells are likely to be the same subset that displays low-intensity class II staining in freshly harvested total thymocytes (~10%; Fig. 2A). Because of their low class II surface expression, these cells may bind too weakly to the magnetic beads to be removed. The surface phenotype of the class II^−-depleted population was primarily CD8^+, CTX^+, class I^− (Fig. 5A), as well as CD5^+ and XT1^+ (data not shown). Depletion of class II^+ cells also removed the small percent of IgM^− (and strongly class II^+) B cells that are typically found in the Xenopus thymus (29).

**Class II^− cells.** Thymocytes depleted of class II^+ cells proliferated poorly in response to PMA/ionomycin (Fig. 6A and Table 1). However, after 3–4 days in culture, a subset of aggregated actively dividing T lymphoblasts once again was detectable. Indeed, by 4 days, this subset accounted for almost 50% of the total number of cells in culture. With or without PMA/ionomycin, the surface phenotype of the subpopulation of small lymphocytes was similar to that seen in cultures of non-depleted thymocytes (Fig. 5A and B). There was still a large percentage CTX^+ and CD8^+ cells. Although in culture treated with PMA/ionomycin, this small lymphocytes population remained class II negative, the entire population of T lymphoblasts clearly expressed high levels of class II molecules (Fig. 5A). Since the starting population was class II^+, these data strongly suggest that rather than selecting for a minor mature population, PMA/ionomycin induced differentiation of a fraction of CTX^+ CD8^+ class II^− immature thymocytes into the more mature and mitotically active class II^+ XT1^+ T lymphoblasts.
subpopulation. [Note that in vivo, the XT1 marker is also expressed by mature peripheral T cells (37).] That differentiation has occurred is further supported by the fact that class II thymocytes were poorly responsive to PMA/ionomycin (Fig. 6A and Table 1).

As with total (non-depleted) thymocytes, the PMA/ionomycin-induced subset of T lymphoblasts also expressed surface class I molecules (Fig. 5A). The expression of class II molecules at the cell surface correlates with the down-regulation of CTX and, to a lesser extent, CD8 molecules.

CTX cells. Similar results were obtained by culturing positively selected CTX thymocytes. For recovering viable CTX cells, panning on anti-CTX mAb-coated culture wells gave better results than magnetic beads. More than 95% of the adherent cells that were recovered after an overnight incubation still expressed surface CTX-like control thymocytes (not shown). Cell death was minimal (>5%) as judged by Trypan blue exclusion and staining with propidium iodide. These CTX cells had a uniform surface phenotype: CD8+ CD5+ XT1+ class I–/class IIlow (Fig. 5B). When these CTX cells were cultured with PMA/ionomycin, thymidine incorporation was even lower than it was for CTX cells selected by removing class II thymocytes (Fig. 6B) and the total live cell recovery after 3 days of culture was low (Table 1). However, a significant cycling T lymphoblast subpopulation (45–50%) expressing high levels of class II molecules was present in culture after 4 days of culture. Most of these T lymphoblasts still expressed surface CD8 molecules, albeit at a low level; they were also surface XT1+. Most importantly, CTX expression was down-regulated by treatment with PMA/ionomycin.

Discussion

PMA/ionomycin induces differentiation of Xenopus immature thymocytes into more mature cycling T lymphoblasts

The present study shows for the first time that sustained proliferation as well as differentiation of Xenopus thymocytes can be obtained in vitro by treatment with a low concentration of PMA plus ionomycin (summarized in Fig. 7).

In mice, low concentrations of PMA plus ionomycin mimic low level stimulation of the TCR–CD3 complex resulting from its interactions with self-peptide presented by MHC molecules (8). Since signaling by PMA bypasses the TCR by directly activating PKC (10), differentiation of thymocytes from TCR- or RAG2-deficient mouse strains that are blocked at the DP stage can be obtained by culturing them with PMA (9,10). In addition, the final step of in vitro differentiation from DP cells to either CD8+ or CD4+ single-positive thymocytes, seems to depend more specifically on the activity level of the calcium-dependent a and/or b PKC isoform rather than on the newly identified calcium-independent PKC-e isoform (10).

In the case of Xenopus, regardless of whether the thymocytes put into culture were unselected or selected, the various concentrations of PMA and ionomycin used (0.2–10 and 200–400 ng/ml respectively) consistently resulted in the
Thymocyte differentiation in Xenopus

Fig. 4. Phenotypic changes of total thymocytes induced by a transient treatment (16 h) with 10 ng/ml PMA and 400 ng/ml ionomycin. Cell phenotype was analyzed before (day 0), directly after extensive washing (day 1) or after thymocytes had been put back in culture without PMA/ionomycin (day 2, 3 and 4). Data are presented as total live cell number starting with $2 \times 10^6$ cells/ml/well. Percent obtained by flow cytometry for the different markers and for the two types of cell subsets (small and lymphoblast) have been used to calculate the respective cell numbers. Gates were set to exclude 95–98% of the cells that stained with non-specific isotype-matched antibodies. For cell proliferation assays ($5 \times 10^4$ cells/wells), cells were pulsed for 20 h with $[^3H]$thymidine after 16 h of PMA/ionomycin treatment and washes or after a further 2 days in culture. Controls cells were cultured in medium alone all the time.

appearance of class II$^+$ class I$^+$ proliferating XT1$^+$ T lymphoblasts. This strongly suggests the induction of differentiation rather than just the selection and the proliferation of a differentiated minor cell population, especially since the lower concentrations of PMA/ionomycin were minimally mitogenic and only a relatively brief exposure of thymocytes was needed. The concentration range of PMA inducing thymocyte differentiation of Xenopus thymocytes appears to be broader than that reported for mice. Although reasons for this are unclear, it may simply be reflective of species variability. In this regard, however, it is important to remember that adult Xenopus differ from rodents at the level of lymphocyte phenotype. For example, all Xenopus peripheral T cells express high levels of surface MHC class II molecules. Although most thymocytes (80–90%) also express surface class II, there is a wide variation in this expression; fluorescence intensity by flow cytometry may vary by $>3$ log (30,33,34,41). At least in young adults, this variation of thymocyte surface class II expression is not uniformly distributed in the thymus. Rather, the pattern of class II staining displays a continuous gradation from a low level in the cortex to a high level in the medulla. It seems reasonable to propose that this distribution is reflective of T cell maturation (34). Whether this difference, together with the broad PMA dose-response of Xenopus thymocytes, are indicative of differences in thymic selection between frog and mouse, remain to be determined.

CTX is down-regulated during differentiation of resting immature thymocytes into cycling T lymphoblasts

Additional observations also reveal similarities between the thymocyte differentiation pattern in mammals and adult Xenopus. For example, as in mammals, CD8 molecules are expressed by the majority of Xenopus thymocytes (70–80%) of the total number of thymocytes as determined by flow cytometry; 23,41). Also as in mice, XenopusCD8$^+$ thymocytes are predominantly localized in the thymic cortex, whereas
Fig. 5. (A) Surface phenotype characterization by flow cytometry class II-depleted (starting population) cultured for 4 days in medium alone (untreated culture) or with 10 ng/ml PMA and 400 ng/ml ionomycin (PMA/ionomycin culture). (B) CTX⁺-selected thymocytes cultured for 4 days in medium alone (untreated culture) or with 10 ng/ml PMA and 400 ng/ml ionomycin (PMA/ionomycin culture). In both (A) and (B), small lymphocyte (1) and larger lymphoblast (2) subpopulations were gated as in Fig. 2. Cells were stained with mAb against Xenopus CTX (X71), CD8 (AM22), class II (AM20) or class I (TB17) molecules. In total, 10,000 events were analyzed on an Elite flow cytometer (Coulter).

Fig. 6. Cell proliferation of purified thymocyte population induced by PMA/ionomycin. (A) Cell proliferation assay (log c.p.m.) of total, MHC class-II enriched or MHC class II-depleted (by magnetic beads) thymocytes cultured in triplicate (2 × 10⁴ cells/wells) for 1, 3 and 6 days in normal medium or in the presence of 10 ng/ml PMA and 400 ng/ml of ionomycin. (B) Cell proliferation assay of total, CTX⁺ (panned on anti-CTX-coated Petri dishes) and CTX⁻ thymocytes (5 × 10⁴ cells/wells) for 1, 3 and 6 days in normal medium or in the presence of 10 ng/ml PMA and 400 ng/ml of ionomycin. In all experiments, cells were pulsed for the last 20 h with [³H]thymidine.

MHC class I⁺ thymocytes are found in the medulla (30,33,34). Although there is ample functional evidence in Xenopus for T₈ cells and for negative selection in the thymus (42,43), the current lack of anti-Xenopus CD4 mAb makes it impossible to categorically state that Xenopus CD8⁺ thymocytes are also CD4⁺ (i.e. are in the DP stage of development). In this regard, the recently characterized CTX marker provides new perspective.

Although CTX was first found in Xenopus, a CTX homolog has been now identified in chickens (25,26) and mammals (44). Such phylogenetic conservation, together with a wealth of structural data (45,46), strongly supports the idea that CTX is a member of a new family of cell surface receptors. Like CTX, the chicken homolog, ChT1, which is preferentially expressed by thymocytes (25) and newly emigrated peripheral T cells (26), appears to play a role in T cell differentiation and/or maturation. Thus far in Xenopus, CTX expression appears restricted to cortical class I⁺ thymocytes (23), a population that in mammals corresponds to DP thymocytes (47). Although there is a considerable overlap between the fraction of CTX⁺ and class II⁺ characterized by flow cytometry, panning and depletion experiments suggest that a sub-
very little is known about the function and the regulation of class II during Xenopus thymocyte differentiation. For example, it remains to be determined whether some CTX + thymocytes or immature thymocytes express surface class II at a high level and whether PMA/ionomycin treatment has a direct effect on class II surface expression. Nevertheless, there is clearly a distinct spatial distribution of these two markers on tissue section—anti-class II mAb stains mainly the medulla (30,33,34) and anti-CTX the cortex (23,40). In addition, cell surface expression of CTX on thymocytes during ontogeny can first be detected by immunocytochemistry and flow cytometry at 8 days post-fertilization, ~1 day after CD8 + cells first appear (40). This close ontogenetic relationship between CD8 and CTX expression, as well as the absence of MHC class I, and absent or weak class II surface expression by cortical thymocytes, further supports the idea that CTX + thymocytes are immature.

CTX displays the structural features of a homodimeric receptor that is independent from the TCR (13). Although in vivo CTX is only expressed by a cell subset that is likely to be non-cycling [the majority of DP mammalian thymocytes are resting cells (3)], surface CTX is also strongly expressed by several stable Xenopus lymphoid tumor cell lines derived from spontaneous thymic tumors. Cross-linking CTX on these tumor cells with anti-CTX mAb causes them to divide abnormally, accumulate in the G2/M phase of the cell cycle and become multinucleated. This suggests that CTX may play a role in the control of the cell cycle (27). The induction of proliferation and differentiation of thymocytes in vitro by PMA/ionomycin provides us with a way to further investigate the biological role of CTX in normal thymocytes. This study reveals the following. (i) PMA/ionomycin induces thymocytes (either total or selected populations) to differentiate from small resting CD8 + class I – class II + cells into large, actively cycling class I + class II + CD8low T lymphoblasts. This differentiation process closely correlates with a down-regulation of CTX mRNA and CTX protein at the cell surface, (ii) Class II-depleted or CTX + thymocytes are significantly less responsive (lower yield of thymidine incorporation) than CTX – thymocytes to PMA/ionomycin in terms of induced proliferation. Since cycling CD8low, class I + class II + T lymphoblasts ultimately differentiate from CTX + -selected thymocytes, this suggests that CTX delays proliferation rather than interferes with the differentiation process itself. The possible involvement of CTX in the regulation of the cell cycle is also supported by the fact that cells panned with anti-CTX mAb consistently proliferated less than CTX – thymocytes that were selected by the removal of class II + cells. As with tumor cell lines (27), this cross-linking did not cause cell death as determined by Trypan blue exclusion or flow cytometry.

In summary (Fig. 7), our data suggest that a pool of immature thymocyte precursors with a class II –/class II low CD8 + pan-T cell + IgM – CTX + phenotype can be committed in vitro into more mature cycling T lymphoblasts class I + and class II –. These results are also in agreement with the idea that CTX is involved in controlling proliferation during the differentiation and selection of cortical thymocytes.

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**Abbreviations**

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<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>CTX</td>
<td>Cortical thymocyte antigen in Xenopus</td>
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<td>DP</td>
<td>Double-positive</td>
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<td>PKC</td>
<td>Protein kinase C</td>
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</table>

**References**

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Kong, F., Chen, C. and Cooper, M. D. 1998. Thymic function can be accurately monitored by the level of recent T cell emigrants in the circulation. Immunity 8:97.


