Induction of the CD4$^-$8$^+$ Suppressor Phenotype in CD4$^+$8$^+$ Human Thymocytes by Phorbol Myristate Acetate

YUKIO SAKIYAMA, AKIHITO ISHIZAKA, TOHRU WATANABE, TADASHI ARIGA and SHUZO MATSUMOTO

Department of Pediatrics, Hokkaido University School of Medicine, Sapporo 060

Human thymocytes were separated according to differential agglutination by peanut lectin (PNA). In the fractions obtained, the distribution and quantitative expression of CD antigens was determined by two-color fluorescence flow cytometry. A major population of CD4$^+$8$^+$ and minor populations of CD4$^+$8$^-$ and CD4$^-$8$^+$ cells existed in unfractionated thymocytes. Agglutination of human thymocytes by PNA led to a highly effective enrichment of the CD4$^+$8$^+$ phenotype in the PNA$^+$ thymocytes, and left three distinct phenotypes, CD4$^+$8$^+$, CD4$^+$8$^-$, and CD4$^-$8$^+$, in the PNA$^-$ thymocytes. After treatment of the CD4$^+$8$^+$ cells in both PNA$^+$ and PNA$^-$ fractions for 20 hr with phorbol 12-myristate 13-acetate (PMA), 60%–90% of the cells expressed the CD4$^+$8$^+$ phenotype, whereas the CD4$^+$8$^+$ phenotype was decreased to 1%–3% of the population. In addition, pretreatment of both PNA$^+$ and PNA$^-$ thymocytes with PMA induced suppressor activity in these cells, as shown by inhibition of immunoglobulin secretion by pokeweed mitogen (PWM)-stimulated peripheral blood lymphocytes (PBL).

By using monoclonal antibodies to distinct human T cell surface antigens it has been demonstrated that three major compartments of thymic differentiation exist in humans with a clear correspondence to different anatomical localizations (Reinherz and Schlossman 1980). Stage I thymocytes lack mature T cell antigens but express T9 and T10 antigens. The cells in this compartment account for 10% of all thymocytes and are localized in the thymic cortex. The majority of cortical cells (stage II) coexpress T4 and T8 antigens without T3 antigen, and account for 70% to 80% of all thymocytes. Stage III thymocytes express T3 with either T4 or T8 antigens, and are located in the thymic medulla. Yet, it has also been
demonstrated that thymocytes display a high degree of phenotypic heterogeneity regardless of their anatomical localization (Umiel et al. 1982; Gelin et al. 1984). Recent studies using multi-color fluorescence flow cytometry show that a majority of cortical thymocytes coexpresses CD4 and CD8 antigens, and at least two phenotypic subpopulations, CD3^-4+/8^+ and CD3^+4+/8^+, can be distinguished (Blue et al. 1986a; Lanier et al. 1986). Although it has generally been perceived that immature cells within the thymic cortex develop into mature medullary thymocytes, it has also been shown that the vast majority of CD4^+8^+ cells die in the thymus without ever becoming functional (von Boehmer 1986). It remains to be shown whether or not CD4^+8^+ cells can differentiate into functional CD3^+4^-8^+ and CD3^+4^-8^+ cells.

In mice and humans, peanut lectin (PNA) mainly labels immature cortical thymocytes, whereas more mature medullar thymocytes are not labeled (Reisner et al. 1976; Betel et al. 1980; Richard et al. 1981). Although there have been numerous studies on the effect of phorbol 12-myristate 13-acetate (PMA) on the antigen expression of some T cell lines (Dröge 1986), there is little information regarding the effect of PMA on normal human thymocytes.

In this report, we demonstrate that PMA treatment of the CD4^+8^+ cells in human thymocytes can induce phenotypic transition into CD4^-8^+ cells, and induces suppressor activity affecting immunoglobulin secretion by PBL.

**Materials and Methods**

*Thymus preparations*

Thymocyte suspensions were prepared from fragments of human thymus obtained from children 1-5 years old undergoing open heart surgery. Normal thymus aseptically removed was finely minced into small sections, and a single-cell suspension was obtained by pressing the fragments through a stainless steel mesh. The thymocytes were washed three times with RPMI 1640 (GIBCO, Grand Island, NY, USA) containing 10% fetal calf serum (FCS, GIBCO) and then isolated by Ficoll/Hypaque centrifugation for 30 min at 4°C. After this procedure thymocytes were >90% viable as assessed by trypan blue dye exclusion. Cells at a concentration of 1 x 10^6 cells/ml in RPMI 1640 plus 10% FCS, were incubated with or without the addition of 10 nM phorbol 12-myristate 13-acetate (PMA, Sigma Chemical Co., St. Louis, MO, USA) at 37°C, 5% CO₂. After 20 hr, cells were washed and stained for analysis by two-color fluorescence flow cytometry.

*Reagents.* Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (MAb) Leu-2a and Leu-4, and phycoerythrin (PE)-conjugated MAb Leu-3a and IL-2R, were purchased from Becton Dickinson Immunocytochemistry Systems. All MAb were assayed for specificity and optimal titer before use. Concanavalin A (Con A) and peanut agglutinin (PNA) were obtained from Sigma Chemical Co., and pokeweed mitogen (PWM) was from GIBCO.

*Immunofluorescence analysis.* Thymocytes (1 x 10⁶) in 50 ul of cold phosphate-buffered saline (PBS) were placed into 12 mm × 75 mm plastic tubes. Optimal amounts of FITC-conjugated and PE-conjugated MAb were added simultaneously to the appropriate tubes, and the samples were vortexed. Samples were incubated for 30 min at 4°C and analyzed on an Epics C cell sorter (Colter Electronics Inc., Hialeath, FL, USA) equipped with an Argon ion laser.
Induction of the CD4+8+ in CD4+8+ Human Thymocytes by PMA

Separation of agglutinated (PNA+) and non agglutinated (PNA−) thymocytes by PNA

These separations were performed as described by Reisner et al. (1976). In brief, thymocytes were suspended at 1 x 10⁶ cells/ml in Hanks’ balanced salt solution (HBSS) and an equal volume of PNA (1 mg/ml in HBSS) was added. After 10 min at room temperature, the cell suspension was layered onto 30 ml of 20% FCS in HBSS. Aggregated PNA+ cells settled rapidly. After 30 min, the top 15 ml-fraction containing the PNA− cells was removed with a pasteur pipet and was centrifuged (10 min, 200 x g). The PNA− pellet and the aggregated PNA+ cells were resuspended in 0.25 M galactose adjusted to 305-310 mOsM with 10 x HBSS. The aggregates could be dispersed readily by gentle pipetting.

Suppressor cell activity of thymocytes affecting immunoglobulin secretion of PWM stimulated peripheral blood lymphocytes

Peripheral blood lymphocytes (PBL) from healthy adults were obtained by centrifugation of heparinized blood over Ficoll/Hypaque gradients. T cells were isolated by formation of E rosettes as described previously (Hashimoto et al. 1986). T cells, unfractionated thymocytes, and PNA+ and PNA− thymocyte fractions were incubated with either 10 µg/ml Con A or 10 nM PMA in RPMI 1640 for 48 hr. After this incubation, cells were harvested, washed three times with 0.3 M α-methyl-D-mannopyranoside (α-MM, Calbiochem.), and once with RPMI 1640 to remove surface-bound Con A and PMA. To analyze the induction of suppressor cell activity of treated T cells and thymocytes, either Con A-stimulated or PMA-stimulated cells were added to fresh PBL (5 x 10⁶/ml in RPMI 1640, 10% FCS) with 10 µg/ml PWM. After a 7 day culture period, the supernatant was harvested and assayed for the presence of immunoglobulin by enzyme-linked immunosorbent assay as described previously (Ariga et al. 1985).

RESULTS

Phenotypic expression of antigens on PNA+ and PNA− thymocytes analyzed with monoclonal antibodies

Thymocytes were analyzed by two-color fluorescence flow cytometry for the presence of “double positive” (CD4+8+) cells after reaction with different PE and FITC conjugated MAb. Analyses of CD antigen expression of PNA+ and PNA− thymocytes are shown in Table 1. The majority (about 80%) of PNA+ thymocytes expressed the CD4+8+ phenotype, whereas there were three major

<table>
<thead>
<tr>
<th>Thymocytes</th>
<th>Percentage of positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD 3+</td>
</tr>
<tr>
<td>Unfractionated thymocytes</td>
<td>60</td>
</tr>
<tr>
<td>PNA+ thymocytes</td>
<td>52</td>
</tr>
<tr>
<td>PNA− thymocytes</td>
<td>66</td>
</tr>
</tbody>
</table>

*Immunofluorescence analysis data were obtained from separate experiments with an Epics C fluorescence flow cytometer. Values represent mean values of 6 experiments using different thymus separations.
subpopulations within the PNA- thymocytes, CD4+8+ (44%), CD4+8- (26%), and CD4-8+ (26%). Table 1 also shows that the percentage of double positive cells is greater than the percentage of CD3+ cells within the PNA+ thymocyte fraction.

Interleukin-2 receptor (IL-2R) and CD3 antigen expression by thymocytes after PMA treatment

The expression of IL-2R and CD3 after PMA treatment was analyzed by two-color fluorescence using PE-conjugated anti-CD3 MAb and FITC-conjugated anti-IL-2R MAb (Table 2). Treatment of thymocytes for 20 hr with 10 nM PMA resulted in a reduction of cells expressing CD3 and in an increase of CD3+ IL-2R+ cells. The expression of IL-2R on the CD3+ cells was greater in PNA- thymocytes than in PNA+ thymocytes.

CD4 and CD8 antigen expression by thymocytes after PMA treatment

The coexpression of CD4 and CD8 antigen after PMA treatment was analyzed by two-color fluorescence following labeling of thymocytes with PE-conjugated anti-CD4 MAb and FITC-conjugated anti-CD8 MAb. Fig. 1 shows loss of CD4 antigen expression on CD4+8- and CD4+8+ cells induced by 10 nM PMA treatment of both PNA+ and PNA- thymocytes. The total percentage of CD8+ thymocytes shows no significant change, however, after PMA treatment of both PNA+ and PNA- thymocytes, the majority of cells showed the CD4-8+ phenotype (Table 3). The cell number and viability of the thymocytes after PMA treatment showed no considerable changes.

<table>
<thead>
<tr>
<th>Thymocytes</th>
<th>Percentage of positive cells</th>
<th>Percentage of positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD 3+</td>
<td>CD 3+ IL-2R+</td>
</tr>
<tr>
<td>Unfractionated thymocytes</td>
<td>—</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>PMA</td>
<td>36</td>
</tr>
<tr>
<td>PNA+ thymocytes</td>
<td>—</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>PMA</td>
<td>30</td>
</tr>
<tr>
<td>PNA- thymocytes</td>
<td>—</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>PMA</td>
<td>53</td>
</tr>
</tbody>
</table>

*Percentage of positive cells after treatment with 10 nM PMA for 20 hr were determined by fluorescence flow cytometry. Values represent mean values of 6 experiments using different thymus preparations.
Induction of the CD4⁻8⁺ in CD4⁺8⁺ Human Thymocytes by PMA

Effect of PNA⁺ and PNA⁻ thymocytes in immunoglobulin secretion after PMA treatment

To determine the function of the cells which express the CD4⁻8⁺ phenotype after PMA treatment, thymocytes were pretreated with either 10 nM PMA or 10 μg/ml Con A, and added to cultures of PBL with PWM. The T cell population of PBL showed suppressor activity in PWM-stimulated immunoglobulin secretion after treatment with either Con A or PMA (Fig. 2A). The unfractionated, PNA⁺, and PNA⁻ thymocytes pretreated with Con A showed no suppressor activity in immunoglobulin secretion, whereas all these thymocyte functions pretreated with

![Diagram](image)

Fig. 1. T4 and T8 fluorescence on unfractionated thymocytes after treatment with only medium (A) and PMA (B) for 20 hr. Thymocytes were stained for T4 and T8 fluorescence with FITC-conjugated MAb Leu-2a and PE-conjugated MAb Leu-3a. Samples were analyzed by fluorescence flow cytometry and data were displayed as two-dimensional contour plots.

**Table 3. Phenotypic characterization of unfractionated, PNA⁺ and PNA⁻ thymocytes after PMA treatment**

<table>
<thead>
<tr>
<th>Thymocytes</th>
<th>Percentage of positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD4⁻8⁻</td>
</tr>
<tr>
<td>Unfractionated</td>
<td></td>
</tr>
<tr>
<td>thymocytes</td>
<td></td>
</tr>
<tr>
<td>PMA</td>
<td></td>
</tr>
<tr>
<td>PNA⁺ thymocytes</td>
<td></td>
</tr>
<tr>
<td>PMA</td>
<td></td>
</tr>
<tr>
<td>PNA⁻ thymocytes</td>
<td></td>
</tr>
<tr>
<td>PMA</td>
<td></td>
</tr>
</tbody>
</table>

*Percentage of positive cells after treatment with 10 nM PMA for 20 hr were determined by fluorescence flow cytometry. Values represent mean values of 3 experiments using different thymus preparations.*
PMA profoundly suppressed PWM-stimulated immunoglobulin secretion in a dose-dependent manner to the same extent as T cells of PBL (Fig. 2B, C, D).

**DISCUSSION**

It has been suggested that several major compartments of thymic differentiation exist in humans (Reinherz and Schlossman 1980). Recent studies have demonstrated that the functional activity of thymocytes is acquired at the level of CD3 antigen expression, and that the majority of common thymocytes express both CD4 and CD8 antigens within the CD3+ phenotype (Blue et al. 1986a; Lanier et al. 1986). In this study, we separated thymocytes by agglutination with peanut agglutinin (PNA) and analyzed phenotypic expression by using two-color fluorescence flow cytometry. The majority of thymocytes that bound PNA (PNA+) also expressed double positive (CD4+8+) phenotype, while about 50% of PNA+ thymocytes expressed the CD3 phenotype. This result demonstrates that some of the CD4+8+ cells within the PNA+ thymocyte fraction express no CD3 antigen and belong to the class of immature thymocytes. In agreement with the recent report of Lanier et al. (1986), our results suggest that there are two major subpopulations of PNA+ thymocytes comprised of 30% CD3-4+8+ cells and 50% CD3+4+8+ cells. PNA- thymocytes consisted of about 20% each of the CD4+8- and CD4-8+ phenotypes, and 40% double positive cells. de Vries et al.
Induction of the \textit{CD}4$^-\textit{8}^+$ in \textit{CD}4$^+\textit{8}^+$ Human Thymocytes by PMA

(1983) suggest that small human thymocytes contain $\sim$30\% PNA$^-$ cells and fail to respond to mitogenic lectin. It is currently under investigation whether \textit{CD}3$^+4^+8^+$ cells within PNA$^+$ and PNA$^-$ thymocyte fractions represent different stages of functional maturation in human thymus.

Several reports show that PMA, a potent activator of protein kinase C induces the expression of interleukin-2 receptor (IL-2R), and causes the disappearance of CD3 antigens in a T cell hybridoma line, T cell clones, T cell tumors, and peripheral blood lymphocytes (Della et al. 1982; Ando et al. 1985; Bensussan et al. 1985). We studied the effect of PMA on thymocytes and found that IL-2R expression on CD3$^+$ cells was induced in PNA$^-$ thymocytes after treatment with PMA to a greater extent than in PNA$^+$ thymocytes. We suggest that the induction of IL-2R on CD3$^+$ cells by PMA is dependent on the maturation of the T cells. Our results also suggest that PMA down-regulates the CD4 antigen on human thymocytes. The modulation of the CD4 antigen by phorbol esters in mature peripheral blood lymphocytes and immature small thymocytes has been described previously (de Vries et al. 1983; Jagielski et al. 1983; Hoxie et al. 1986). We demonstrated that the action of PMA reduced the expression of CD4 antigen on CD4$^+8^+$ thymocytes, which led to the expression of the CD4$^-8^+$ phenotype by the majority of PNA$^+$ and PNA$^-$ thymocytes. In contrast to PMA treatment, the treatment with Con A showed neither considerable change of the phenotypes (data not shown) and induction of suppressor activity in the CD4$^+8^+$ thymocytes. To further investigate this phenotypic change, the thymocytes pretreated with PMA were added to PBL cultured with PWM. We found that both PNA$^+$ and PNA$^-$ thymocytes showed suppressor activity in PWM-induced immunoglobulin secretion, to the same extent as pretreated T cells of PBL.

It has been reported that PMA treatment of peripheral blood lymphocytes inhibits immunoglobulin production stimulated by PWM (Jagielski et al. 1983). These authors suggest that the PMA-induced suppression is T cell-dependent, and that the induction is related to proliferation and activation of CD4$^+$ and CD8$^+$ lymphocytes. Our data provide evidence that PMA induces the down-regulation of CD4 antigen and the induction of suppressor cell activity in human thymocytes.

The biological role for the modulation of CD4 antigen in human thymocytes by PMA is still uncertain. It has been found that CD3$^+4^+8^+$ cells represent an intermediate in the maturation of human thymocytes and that the activated CD4$^+8^+$ thymocytes give rise to the CD4$^-8^+$ cell subset (Blue et al. 1986b). Our results showing that PMA down-regulates the expression of the CD4 antigen in CD4$^+8^+$ thymocytes and give rise to suppressor cells suggest that activation of protein kinase C can be correlated with the maturation of T cells in human thymus.

Acknowledgments

This work was supported by grant from the Ministry of Health and Welfare, Japan.
References


