0022-1767/83/1311-0201$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 131, No. 1, July 1983
Printed in U.S.A.

INDUCTION OF PHENOTYPIC DIFFERENTIATION, INTERLEUKIN 2 PRODUCTION, AND PHA RESPONSIVENESS OF "IMMATURE" HUMAN THYMOCYTES BY INTERLEUKIN 1 AND PHORBOL ESTER

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Small human thymocytes (ST) representing 70% of the thymocytes were isolated according to size by centrifugal elutriation. Although these ST contained approximately 30% PNA$^-$ cells, they failed to respond to lectins, indicating the existence of a PNA$^-$ ST subset that can be considered to belong to the "immature" thymocyte population. The ST were induced to proliferate if, in addition to PHA, IL 1-containing supernatants of highly purified monocyte cultures or 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) were present. The incubation of the ST for 90 hr with TPA or IL 1 in the absence of PHA resulted in a strong reduction in the percentage of cells reacting with the immature thymocyte markers TdT and PNA. In addition, the OKT6$^+$ cells were partially reduced after incubation with IL 1. Concomitantly, an increase in the percentage of cells reacting with the mature T cell markers OKT1 and OKT3 was observed, whereas HLA antigens became strongly expressed on all ST. Although IL1 or TPA were unable to induce proliferation of the ST, these substances induced IL 2 production by these cells. These shifts to cells with more "mature" phenotypes that are able to produce IL 2 were not observed if the ST were incubated with PHA or culture medium only. The responder capacity of the ST to PHA plus TPA was not significantly affected by the depletion of the more "mature" OKT3$^+$ and OKT1$^+$ cells. In addition, in this situation OKT1$^+$, OKT3$^+$, OKT6$^-$ cells were found to be generated from OKT1$^-$, OKT3$^-$, OKT6$^+$ cells. Therefore, it could be excluded that the proliferative responses were due to a selective expansion of a preexisting mature T cell population. Our results indicate that TPA mimics IL 1 in the induction of differentiation of the ST to a stage in which subpopulations of these cells are able to produce IL 2 and to respond to PHA. Because only the proliferating ST were found to react with a monoclonal antibody, which is thought to be directed at the IL 2 receptor (anti-Tac), our data suggest that PHA is required for the induction of expression of receptors for IL 2 in those ST subpopulations that are able to proliferate in the presence of IL 2 generated *in situ*.

Monocytes/macrophages have been shown to be required for optimal lectin-induced T cell proliferation *in vitro* (1–8). This monocyte/macrophage function could be replaced in a nonrestricted way by interleukin 1- (IL 1)[2] containing media of monocyte cultures (1, 9, 10) or by 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) (10–14). IL 1 and TPA were also found to be capable of inducing lectin responsiveness of small human thymocytes (ST), which represented 70% of all thymocytes (11, 15). In addition to their lack of lectin responsiveness, these ST failed to respond to alloantigens and therefore were considered to represent an "immature" thymocyte subset. Because it has recently been proposed that interleukin 2 (IL 2) is the only mitogenic signal for T cells (16, 17), and IL 2 production is shown to require the presence of accessory cells, IL 1 produced by these cells (18–20), or TPA (12, 14, 21, 22), it seems likely that the lack of lectin responsiveness of the ST has to be attributed to the failure of these cells to produce IL 2.

It is well established that TPA induces differentiation of a large variety of cells (23) including human T lymphocytes and thymocytes (11, 24, 25). Taken together with the observation that IL 1 can induce thymocyte differentiation (9, 11, 26), these data prompted us to investigate whether the induction of PHA responsiveness of the ST by IL 1 or TPA was the result of the generation of immunocompetent T cells from the "immature" ST. This question is of interest because there is some controversy regarding the contention that all immature cortical thymocytes are able to mature to immunocompetent T cells in the presence of IL 2. Various investigators have shown that immature peanut agglutinin-positive (PNA$^+$) murine thymocytes could differentiate to cytotoxic T cells in the presence of IL 2 (27–30). In contrast, other investigators have excluded IL 2-mediated maturation of PNA$^+$ thymocytes to immunocompetent T cells both in murine (31, 32) and in human systems (33). The recent observations in mice made by Chen *et al.* (32) demonstrated that the frequency of cytolytic precursor cells and precursors of cells capable of proliferation in the PNA$^+$ cell population is low compared to those in the PNA$^-$ subset and probably has to be attributed to contamination with PNA$^-$ cells.

In this paper, we show that both IL 1 and TPA induce phenotypic differentiation of "immature" human ST to a point at which subpopulations of these cells are able to produce IL 2 and to respond to PHA.

MATERIALS AND METHODS

Media and reagents. All cell cultures and tests were carried out in Dulbecco's modification of Eagle's minimal essential medium (DMEM) supplemented with 3.8 g $NaHCO_3$/liter, L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), fungizone (2.5 μg/ml), and 10% heat-inactivated (45 min, 56°C) fetal calf serum, all purchased from GIBCO Biocult (Glasgow, Scotland). This medium was designated as culture medium. Purified phytohemagglutinin (PHA) was purchased from Wellcome, Beckenham, England. TPA, batch 26,

Received for publication September 13, 1982.
Accepted for publication March 10, 1983.
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[2] Abbreviations used in this paper: IL 1, interleukin 1; IL 2, interleukin 2; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; ST, small thymocytes; PNA, peanut agglutinin; TdT, terminal deoxynucleotidyl transferase; FITC, isothiocyanate.

was obtained from Consolidated Midland Corp., Brewster, NY. TPA was dissolved in ethanol at a concentration of 10 mg/0.5 ml and stored at −80°C as a stock solution of 0.4 mg/ml diluted in DMEM without serum. TPA was diluted to the appropriate concentration in culture medium just before use. Affinity-purified goat antibody to terminal deoxynucleotidyl transferase (TdT) (34) was a gift from Dr. F. J. Bollum, Uniformed Services, University of the Health Sciences, Bethesda, MD. Fluorescein isothiocyanate-(FITC) labeled PNA was purchased from L'Industrie Biologique Francaise, Clichy, France (35). Tritiated thymidine (^{3}H-TdR, specific activity 6.7 Ci/mmol) was obtained from New England Nuclear, Boston, MA.

Monoclonal antibodies. Monoclonal antibodies directed against human T cell differentiation antigens were used. The reactivity of the monoclonal antibodies OKT6 (36) and NA 134 (37) is restricted to human thymocytes, whereas OKT1 (38) and OKT3 react with most of the mature T cells (39). OKT4 reacts generally with T cells of the inducer or helper type (40), but also with a subset of cytotoxic T cells (41), whereas OKT8 reacts with the T cell populations that include cells with suppressor and cytotoxic functions (36). W6/32 reacts with the heavy chains of all HLA-A, -B and -C determinants (42). Anti-Tac, which reacts with IL 2-dependent activated T cells but not with resting and IL 2-independent T cells (43, 44) and is thought to recognize the IL 2 receptor (45), was kindly provided by Dr. T. A. Waldmann (Metabolism Branch, NCI/NIH, Bethesda, MD). The binding of these antibodies to viable cells in suspension was detected with FITC-labeled goat anti-mouse Ig. Fluorescence staining was evaluated by means of a fluorescence microscope as described (46).

Separation of thymocytes. Normal human thymus was obtained from children from the ages of 8 mo to 6 yr undergoing corrective cardiac surgery (kindly provided by Dr. H. J. Schuurman, University Hospital, Utrecht, The Netherlands). The fresh thymic fragments were collected in culture medium and carefully minced. Single thymocyte suspensions were prepared by pressing through a 200-mesh stainless steel mesh. The thymocyte suspensions were separated according to the size by a modified centrifugal elutriation procedure described previously (15). Approximately 70% of the thymocytes were elutriated at a constant flow rate of 18 ml/min and a speed of rotation of 3000 rpm. This fraction contained a homogeneous population of ST judged by electronic sizing and cytocentrifugal preparations stained with May-Grünwald-Giemsa stain. Accessory cells detected by staining for nonspecific esterase were absent. The viability of the thymocyte fractions was over 95% in all instances, determined by trypan blue exclusion.

Thymocyte cultures. 3×10^5 thymocytes were seeded in microtiter plate wells (no. 3040; Falcon Plastics, Oxnard, CA) in a final volume of 0.2 ml. PHA and TPA were added at concentrations of 1 and 0.1 μg/ml, respectively, which were shown to be optimal (10). IL 1 was added at concentrations that have been shown to have the strongest mitogenic effects on murine thymocytes (12.5 to 25% v/v). The cultures were incubated for 4 days, pulsed for 3 h with 0.4 μCi ^{3}H-TdR, and harvested and counted as described (6).

Preparation of crude IL 1. In the first series of experiments, IL 1-containing supernatants were obtained from monocytes activated with TPA. Human monocytes were isolated by centrifugal elutriation of buffy coat cells obtained from 1 unit (500 ml) of blood (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam) as described (47). These purified monocyte fractions contained 95% monocytes. Then 2×10^6 monocytes were cultured in Linbro plates (No. 76-033-05; Linbro Chemical Co., New Haven, CT) in the presence of 0.1 μg/ml TPA and 2.10^{-5} M indomethacin in a final volume of 2 ml. The 24-hr supernatants of these activated monocytes were collected, centrifuged at 1500 × G for 10 min, and were used as crude IL 1 source. TPA was removed sufficiently from these culture supernatants by Millipore filtration (10, 48); culture medium in which TPA (0.1 μg/ml) had been incubated for 24 hr in the absence of monocytes failed to induce a proliferative response of the ST to PHA.

Most experiments, however, were carried out with IL 1 obtained from monocytes cultured on microcarrier beads (49) in serum-free medium (41) in the absence of TPA. The Cytodex microcarrier beads (Pharmacia Fine Chemicals, Uppsala, Sweden) were swollen in phosphate-buffered saline (PBS) for 24 hr, washed five times with PBS, and autoclaved. After autoclaving, the beads were washed three times with PBS and resuspended in serum-free medium at a concentration of 100 mg/ml. The wells of the Linbro plates were filled with 250 μl microcarrier bead suspension and subsequently with 4×10^6 monocytes in the presence of 2×10^{-5} M indomethacin. The final volume of the wells was 2 ml. After 24-hr incubation at 37°C and 5% CO_2, the culture supernatants were collected by centrifugation at 1500 × G for 10 min and used as IL 1 source. Both crude IL 1-containing preparations were directly mitogenic for mouse thymocytes and enhanced the PHA-induced proliferation of murine and human thymocytes. Optimal effects were observed at concentrations of 12.5 to 25% (v/v). In addition, the IL 1 preparations did not contain interferon activity determined by neutralization of the cytopathic effect of vasicular stomatitis virus on murine L cells or IL 2 activity (measured as described below).

Assay for IL 2 activity. The capacity of IL 2 produced by the thymocyte cultures to maintain T cell proliferation was tested as described by Gillis *et al.* (50) for murine IL 2. The IL 2 activity was tested on a murine T cell line (TR) kindly provided by Dr. L. Aarden (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), which was shown to be dependent on human IL 2 purified by high-pressure liquid chromatography and isoelectric focusing. TR cells (5×10^3) were cultured in 200-μl vol in flat-bottomed microplate wells in DMEM culture medium in the presence of a $\log_2$ dilution (ranging from 0.25 to 50% (v/v)) of a putative IL 2-containing thymocyte culture supernatant. After 24-hr incubation at 37°C and 5% CO_2, the cultures were pulsed with 0.4 μCi ^{3}H-TdR, harvested 4 hr later, and counted as described (6). IL 2 activity was quantitated by probit analysis with the use of a 10% human IL 2 sample prepared from PHA-stimulated peripheral blood leukocytes of three different donors as standard, arbitrarily assigned a value of 100 U/ml. The PHA and/or TPA used for the stimulation of the thymocyte cultures did not interfere with the assay because the culture medium in which PHA and/or TPA had been incubated for 3 or 4 days had no proliferative effect on the TR cells. In addition, the TR cells did not respond to IL 1-containing supernatants of monocytes cultured in the presence of TPA or on microcarrier beads.

Depletion of OKT-3$^+$ thymocytes. 50×10^6 ST were collected in a volume of 0.2 ml of PBS supplemented with 0.5% bovine serum albumin (BSA) and incubated with 0.8 ml OKT3, 1/200 diluted in PBS for 60 min at 4°C with occasional shaking. After this incubation period, 0.2 ml of a selected batch of nontoxic 1/10 diluted rabbit serum was added as a C source, and the cell suspension was incubated for another hour in a 37°C waterbath with shaking. The cells were washed with PBS plus 0.5% BSA and counted. The recoveries varied between 30 to 50% and the viability between 70 to 95%.

RESULTS

Differentiation state of ST incubated with TPA or IL 1 in the presence or absence of PHA. We previously showed that ST isolated by centrifugal elutriation failed to respond to PHA, but that proliferation was induced if, in addition to PHA, irradiated allogeneic monocytes, crude IL 1 preparations, or TPA were added (11). Because mitogen responsiveness is a property of mature medullary thymocytes and both IL 1 and TPA can induce thymocyte maturation differentiation (9, 11, 26), we investigated whether the induced proliferation of the ST was a result of differentiation/maturation. The differentiation state of the ST before and after incubation with IL 1 or TPA in the presence or absence of PHA was determined by their reactivity with monoclonal antibodies detecting T cell differentiation antigens and HLA-A, -B, and -C antigens, and by their reactivity with PNA and TdT. The phenotype of the ST was not significantly different from that of the corresponding unfractionated thymocytes (Table I). In spite of their immature phenotype, 20 to 50% of the cells in the ST population were found to react with the mature T cell markers OKT1 and OKT3. Interestingly, the ST also contained approximately 30% small PNA$^-$ thymocytes that, because of their lack of PHA responsiveness, can be considered to belong to the immature thymocyte population.

Incubation of the ST with TPA or IL 1 did not result in proliferation (Table II), but some degree of differentiation was observed, shown by the strong decrease in the percentage of cells reacting with PNA, TdT, and OKT4; these markers were still present on the ST incubated with culture medium or with PHA. In contrast to the ST incubated with IL 1, however, those incubated with TPA showed a considerable reduction in the OKT6$^+$ and NA 134$^+$ cells. The percentage of OKT8$^+$ cells was only slightly reduced. Furthermore, a strong increase in the percentage of cells expressing HLA antigens was observed, whereas the fluorescence intensity of these cells was also strongly increased (not shown). The percentages of OKT1$^+$ and OKT3$^+$ cells were significantly enhanced. The increase of the OKT3$^+$ cells after incubation with TPA could only be observed after the cells had been washed and incubated overnight in culture medium, suggesting that the binding of OKT3 or expression of the OKT3 antigen was affected by TPA. Only the proliferating ST were found to react with the anti-Tac antibody, suggesting that in addition to IL 1 or TPA, PHA is required for the expression of the receptors for IL 2. In these proliferating cell populations, both blasts and non-blasts were Tac$^+$ (not shown).

The simultaneous addition of IL 1 and PHA or TPA and PHA

TABLE I

Phenotypic characterization of the unfractionated thymocytes and the corresponding ST before and after incubation with TPA or in the presence or absence of PHA[a]

Antibodies	Unfractionated Thymocytes	ST	ST after 90 hr incubation with					
			TPA	IL 1[b]	PHA + TPA	PHA + IL 1	PHA[b]	Medium
OKT1	29 ± 7	22 ± 3	50 ± 4	57 ± 4	67 ± 5	66 ± 4	18 ± 5	19 ± 2
OKT3	36 ± 6	30 ± 4	53 ± 3[c]	46 ± 4	52 ± 8[c]	58 ± 4	27 ± 5	34 ± 3
OKT4	84 ± 9	76 ± 7	14 ± 4	12 ± 3	9 ± 3	5 ± 2	71 ± 11	65 ± 9
OKT6	87 ± 4	90 ± 5	90 ± 3	43 ± 4	13 ± 1	6 ± 2	78 ± 6	83 ± 4
NA 134	82 ± 3	92 ± 3	94 ± 2	30 ± 5	8 ± 2	4 ± 1	NT	NT
OKT8	86 ± 8	83 ± 5	70 ± 8	75 ± 6	72 ± 5	76 ± 5	67 ± 10	80 ± 7
W6/32	37 ± 5	28 ± 5	96 ± 1	98 ± 1	92 ± 5	86 ± 14	25 ± 6	35 ± 5
PNA	66 ± 8	71 ± 6	22 ± 5	15 ± 5	32 ± 4	35 ± 3	75 ± 8	61 ± 5
TdT	85 ± 7	92 ± 4	0	0	NT	NT	60 ± 15	71 ± 8
Anti-Tac[d]	NT	0	2 ± 1	1 ± 1	32 ± 5	64 ± 5	4 ± 1	0
% Recovery[e]			26 ± 4	30 ± 3	73 ± 9	60 ± 7	32 ± 4	35 ± 3

[a] TPA was added at a concentration of 0.1 μg/ml, PHA at 1 μg/ml, and IL 1 at a concentration of 12.5% (v/v). In two experiments, supernatant of monocytes activated with TPA (0.1 μg/ml) was used as IL 1 source. This IL 1 preparation was mitogenic for BALB/c thymocytes (2.1×10^3 cpm) and enhanced the PHA response of these cells 20.6 times at concentrations of 25% (v/v). The other experiments were carried out with supernatant of monocytes cultured on microcarrier beads as IL 1 source. The latter induced a BALB/c thymocyte proliferation of 1.7×10^3 cpm and enhanced the PHA responses of these cells 44.1-fold. Percent positive cells (mean ± SEM of four experiments).

[b] Mean ± SEM of three experiments.

[c] The percent of $OKT3^+$ cells after washing and resting overnight; direct determination: TPA: 13 ± 3, PHA + TPA: 22 ± 8.

[d] Mean ± SEM of two experiments.

[e] The percent recovery compared with the number of viable cells seeded per well = 100%.

TABLE II

^{3}H-TdR incorporation of the ST and corresponding unfractionated thymocytes after incubation with TPA or IL 1 in the presence or absence of PHA[a]

	PHA	TPA	IL 1	PHA + TPA	PHA + IL 1
Unfractionated thymocytes	19.0 ± 4.4[b]	4.3 ± 0.6	1.6 ± 0.5	70.5 ± 20.1	83.8 ± 17.3
ST	0.4	0.4	0.3	21.4 ± 3.1	18.0 ± 4.2

[a] TPA was added at a concentration of 0.1 μg/ml and PHA at 1 μg/ml. Supernatant of monocytes cultured on microcarrier beads was used as IL 1 source at a concentration of 25% (v/v).

[b] ^{3}H-TdR incorporation cpm × 10^3 (mean ± SEM of three experiments).

resulted in proliferation of the ST (Table II). Characterization of the proliferating ST revealed a further shift to a more "mature" phenotype because in addition to a decrease in TdT^+ and $OKT4^+$ cells, a strong reduction in the percentages of $OKT6^+$ and $NA\ 134^+$ cells was observed (Table I). The percentages of cells reacting with W6/32, OKT1, and OKT3 were also considerably increased. Surprisingly, compared with the ST incubated with TPA or IL 1 only, these proliferating cells contained higher percentages of PNA^+ cells. It should be noted that considerable cell death occurred during the incubation period; however, the recoveries of the surviving cells incubated with culture medium, PHA, IL 1, or TPA did not differ significantly. Therefore these results suggest that IL 1 or TPA can induce some degree of phenotypic differentiation of a proportion of the ST.

Induction of proliferation of $OKT1^+$- and $OKT3^+$-depleted ST. To exclude the possibility that proliferation induced by IL 1 and PHA or TPA and PHA was due to selective outgrowth of the more "mature" $OKT1^+$ and $OKT3^+$ thymocytes present in the ST, the ST were depleted of $OKT3^+$ cells by lysis in the presence of C (Table III). Depletion of $OKT3^+$ cells resulted in a concomitant depletion of $OKT1^+$ cells. These $OKT1^+$- and $OKT3^+$-depleted ST responded almost equally well to PHA in the presence of TPA as did the corresponding non-$OKT3^+$-depleted cell population in the presence or absence of C. Approximately 50% $OKT1^+$ and $OKT3^+$ cells were detected in the proliferating ST, initially depleted of cells expressing these mature T cell antigens. It seems unlikely that the loss of $OKT3^+$ cells was due to antibody-mediated modulation of the OKT3 antigen, because the $OKT1^+$ cells were concomitantly removed by the antibody and C treatment. In addition, 50 to 70% of the ST were found to be removed by this treatment. These results indicate that $OKT1^-$, $OKT3^-$, $OKT6^+$ thymocytes under the influence of PHA and TPA can be induced to proliferate and that the proliferating cells differentiate to a "more mature" $OKT1^+$, $OKT3^+$, $OKT6^-$ phenotype.

IL 2 production by ST. Recently it was shown that lectin-induced T cell proliferation requires both an induction signal provided by IL 1 and a mitogenic signal from IL 2 (14). In addition, because TPA was found to mimic the signal provided by IL 1 (11), we measured the IL 2 production of the ST after incubation with IL 1 or TPA in the absence or presence of PHA.

Although TPA or IL 1 in the absence of PHA did not induce proliferation, significant activity levels of IL 2 were found to be produced by these cells (Table IV). IL 2 production was also observed if IL 1 or TPA were added in the presence of PHA. In this latter situation, however, lower IL 2 activity was detected in

TABLE III

Phenotyping and ^{3}H-TdR incorporation of ST and ST depleted of $OKT3^+$ cells, before and after proliferation induced with PHA (1 μg/ml) and TPA (0.1 μg/ml)[a]

	Fresh		After Proliferation with PHA and TPA	
	ST	ST depleted of $OKT3^+$ cells	ST	St initially depleted of $OKT3^+$ cells
% $OKT3^+$	34 ± 4	<3	51 ± 5[b]	47 ± 3[b]
%$OKT1^+$	23 ± 3	<3	60 ± 4	54 ± 4
% $OKT6^+$	88 ± 4	91 ± 4	9 ± 3	15 ± 2
^{3}H-TdR incorporation cpm × 10^3			22.4 ± 3.2	17.8 ± 5.0

[a] ^{3}H-TdR incorporation (cpm × 10^3) of ST and ST initially depleted of $OKT3^+$ cells in the presence of complement was 20.9 ± 2.8 and 18.5 ± 4.2, respectively (mean ± SEM of two experiments).

[b] Measured after washing and resting overnight in culture medium at 37°C and 5% CO_2.

TABLE IV

IL 2 production of ST after 4 days incubation[a]

Supernatants of ST Incubated with	IL 2 Activity (U/ml)
PHA (1 μg/ml)	0
TPA (0.1 μg/ml)	30 ± 3
IL 1 (12.5% v/v)	28 ± 4
PHA (1 μg/ml) + IL 1 (12.5% v/v)	13 ± 2
PHA (1 μg/ml) + TPA (0.1 μg/ml)	10 ± 4
Reference IL 2	100

[a] Forty-eight hour supernatant of PHA-stimulated PBL from three different donors was used as reference (mean ± SEM of three experiments).

the supernatants, which probably could be attributed to IL 2 consumption by the proliferating cells (14). The possibility that the growth-promoting activity of ST supernatants was due to PHA or TPA could be excluded (see *Materials and Methods*). In addition, the IL 1 preparations used for the activation of the ST were not found to have any detectable IL 2 activity.

These results indicate that ST incubated with IL 1 or TPA, in contrast to those incubated with culture medium or with PHA, mature to a point at which a proportion of these cells can produce IL 2.

DISCUSSION

The present results confirm and extend our previous findings demonstrating that ST fail to respond to PHA. Because the ST contained approximately 30% small, PNA^- cells, these data indicate the existence of a PNA^- immature thymocyte subset. The ST could be induced to proliferate by the addition of IL 1-containing supernatants of highly purified monocytes or TPA (11). it is shown that the lack of PHA responsiveness has to be attributed to the failure of the ST to produce IL 2, which is proposed to be only a mitogenic signal for T cells (16, 17). The finding that IL 2 production by the ST was induced by the addition of exogenous IL 1 indicates that the lack of IL 2 production by the ST probably has to be attributed to the absence of accessory cells in these cell preparations. These data are in line with those of other investigators who demonstrated that IL 2 production only occurred in the presence of accessory cells of IL 1 produced by these cells (18–20). Furthermore, TPA that can replace macrophage/monocyte functions in lectin-induced T cell proliferation (10–14) and IL 2 production (12, 14, 21, 22) was also found to be capable of inducing IL 2 production by the ST. Both TPA (that is mitogenic for human T cells (11, 24, 25) and mature thymocytes (11)) and IL 1, however, failed to induce proliferation of the ST in spite of their capacity to induce significant IL 2 production by these cells. Recently, Stadler *et al.* (14) reported that IL 2 production became detectable as lymphocytes stimulated by exogenous stimulants entered the later phase of G_1. This would imply that IL 1 and TPA induce the ST to shift from the G_0 into the late G_1 phase. Proliferation of the ST was only observed if IL 1 or TPA was added in combination with PHA. In addition, in contrast to the ST incubated with PHA, IL 1, or TPA only, the proliferating ST were found to bind the anti-Tac monoclonal antibody, which is thought to react with the receptor for human IL 2. These results suggest that PHA was required for the induction of expression of IL 2 receptors in those subpopulations present in the ST that are able to proliferate in the presence of IL 2 generated *in situ*. The possibility that the proliferation-maintaining capacity of IL 2 produced by the ST was due to some unknown effect of human proteins on the mouse T cells used for IL 2 determination could be excluded, because similar growth-promoting effects were observed on IL 2-dependent human T cell cultures (results not shown). Recently Maizel *et al.* (9) showed that human thymocyte mitogenesis was strongly enhanced in the presence of partially purified IL 1. Our results indicate that the proliferative responses to PHA induced by IL 1 or TPA are actually mediated by IL 2.

Although both IL 1 and TPA were unable to induce proliferation in the absence of PHA, it was shown that TPA mimicked IL 1 in the induction of differentiation of the ST. The immature thymocyte markers PNA and TdT were strongly reduced after 90 hr incubation with TPA or IL 1. Surprisingly, an increase in the PNA^+ cells was observed in the proliferating ST that were cultured with IL 1 or TPA in the presence of PHA. These data are, however, in line with the observations of Schrader *et al.* (51) who showed that the majority of PNA^- murine thymocytes or lymph node cells change to PNA^+ cells upon activation, which indicates that activated T cells acquire PNA-binding capacity. Furthermore, both TPA and IL 1 induced a strong reduction of the $OKT4^+$ cells that was even more pronounced in the ST cultures that were induced to proliferate in the presence of PHA. The percentage of $OKT8^+$ cells was only slightly reduced, demonstrating that both the proliferating and nonproliferating cells were predominantly restricted to the $OKT8^+$ phenotype. Similar observations were made by other investigators. Hayward *et al.* (52) showed that human thymocytes activated with PHA in the presence of IL 2 belonged to the $T3^+$, $T8^+$ subset. Delia *et al.* (53) reported that leukemic cell lines that were phenotypically identical to normal cortical thymocytes lost their reactivity with TdT, PNA, OKT4, and OKT6 upon Incubation with TPA, whereas the percentage of $OKT8^+$ cells remained unaltered. In our experiments, IL 1 induced a partial reduction of the cells reacting with the thymocyte markers OKT6 and NA 134, whereas TPA had no effect. The $OKT6^+$ and $NA\ 134^+$ cells were strongly reduced, however, in the proliferating ST cultures. The TPA- or IL 1-induced phenotypic modifications also included an increase in the cells expressing HLA antigens and the mature T cell markers OKT1 and OKT3. Taken together, these changes in phenotype suggest that incubation of the ST with TPA or IL 1 induced some degree of maturation. In spite of the considerable cell death that occurred during the incubation period (25 to 35% of the ST survived), it could be excluded that the observed differentiation was due to a selective survival of cells with a more mature phenotype. First, shifts to a more mature phenotype were not observed if the ST were incubated in culture medium only or in the presence of PHA, whereas the recoveries of viable ST after 90 hr incubation in culture medium or PHA were comparable to those incubated with IL 1 and TPA. Secondly, TPA and IL 1 induced IL 2 production, whereas incubation of the ST with culture medium or PHA failed to do so. In addition, the possibility that the PHA responsiveness induced by IL 1 or TPA was due to the selective outgrowth of the more mature $OKT3^+$ thymocytes could be excluded, because no clear reduction in the degree of proliferation could be observed in ST populations depleted for $OKT3^+$ cells, whereas in this situation $OKT1^+$, $OKT3^+$, $OKT6^-$ cells were found to be generated from $OKT1^-$, $OKT3^-$, $OKT6^+$ cells. These data are in contrast to those of Hayward *et al.* (52) who reported that the proliferative responses of human thymocytes induced by PHA in the presence of IL 2 were greatly reduced after depletion of $OKT3^+$ cells. These differences in results are difficult to explain, but may be due to the differences in culture conditions and the fact that TPA or our IL 1 source are more effective in inducing maturation than their exogenous IL 2 source.

Our results, which indicate that both IL 1 and TPA induce maturation of the ST to a point at which subpopulations of these cells are capable of producing IL 2 and to respond to PHA, are in line with our previous observations indicating that TPA induced malignant T cell populations to differentiate and to produce IL 2 (46). Similar findings, which indicate that immunologically unresponsive thymocytes can be induced to differentiate to functionally active mature T cells, were also obtained with murine thymocytes. Various investigators reported that immature cortical thymocytes isolated according to their reactivity with PNA could differentiate to mature cytotoxic T cells in the presence of IL 2 (27–30). Contrasting results, however, have been obtained by other investigators. Bödeker *et al.* (31) excluded IL 2-mediated maturation of PNA^+ murine thymocytes because these cells could not be induced to become responsive to concanavalin A.

Piantelli *et al.* (33) came to the same conclusion after investigating the PHA responsiveness of PNA^+ human thymocytes. Recently Chen *et al.* (32), with the use of murine thymocytes, demonstrated by limiting dilution assays that the frequency of cytolytic precursor cells and the precursors of cells capable of proliferative responses in the PNA^+ cell population were low compared with those in the PNA^- subset. Similar results were reported by Ceredig *et al.* (54) who found that all cytolytic and helper precursor cells were present in the mature medullary thymocyte subset. These data suggest that PNA^+ murine thymocytes differ in maturation stage from our ST populations (separated according to size) in spite of the fact that both populations are considered to be "immature" because of their lack of mitogen and alloantigen responsiveness. This difference in maturation stage may be reflected by the 25 to 40% of small PNA^- cells present in the ST population, but because these PNA^- thymocytes are functionally immature and are therefore different from PNA^- medullary thymocytes, it seems fair to conclude that PNA binding is not an unambiguous marker for immature human thymocytes. This notion is supported by the data of Umiel *et al.* (55) who demonstrated that the PNA^+ human thymocyte subset was heterogeneous in its response to PHA and IL 2. Only PNA^+, $OKT1^+$ thymocytes responded well to PHA in the presence of IL 2, whereas PNA^+, $OKT1^-$ thymocytes remained unresponsive to PHA and IL 2. In addition, it cannot be excluded that PNA binding (that is required for the separation) may affect the functional properties of PNA^+ thymocytes. The functional properties of the PNA^- thymocyte subset present in the ST are currently under investigation.

Acknowledgments. The authors thank Trees Dellemijn and Martien Poelen for their excellent technical assistance, and Marie Anne van Halem for her secretarial help.

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