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Biochemical and immunological differentiation of human thymocytes induced by thymic hormones

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Summary. Changes in levels of purine degradative enzymes have been shown to occur during T-cell maturation in both rats and humans with a fall in adenosine deaminase (ADA) and a rise in purine nucleoside phosphorylase (PNP) and 5′-nucleotidase (5′NT) activities. We have investigated the effects of four thymic factors: thymosin fraction 5 (TMS-F5); thymosin α1 (TMS-α1); thymopoietin pentapeptide (TP-5); and thymic conditioned medium (CM) on TdT activity, purine enzyme levels and the phenotypic markers OKT3 (a marker for mature T cells) and NA1/34 (which reacts with immature cortical thymocytes) in human thymocytes and in the lymphoid leukaemic cell lines RPMI-8402 and JM1 (derived from Thy-ALL). All four thymic factors caused one or more maturation change in human thymocytes, e.g. TMS-F5 caused a significant increase in OKT3 expression, TMS-α1 a fall in TdT and ADA activities and a rise in OKT3-positive cells, TP-5 an increase in PNP and CM a rise in 5′NT activity. TMS-F5 also caused a marked elevation of 5′NT in both the T lymphoblastic lines ($P < 0{\cdot}001$). On the other hand the non-physiological phorbol ester, 12-O-tetradecanoyl phorbol acetate (TPA), a tumour promotor with potency of inducing differentiation in some leukaemic cell lines, induced changes in both normal thymocytes and in the leukaemic line JM1 were inconsistent with maturation, e.g. a fall in the percentage of OKT3 cells.

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These observations suggest that maturation of normal thymocytes might proceed stepwise, each step requiring at least one of the thymic hormones. Although thymosin also induces differentiation changes in a malignant lymphoid line, the pattern of these differs from that induced in their normal counterparts.

INTRODUCTION

It appears likely that the thymus produces a group of hormone-like peptides which control the development of the T lymphoid system (Trainin, Pecht & Handzel, 1983). Thymosin fraction 5 (TMS-F5), the most extensively investigated, has been found to induce expression of terminal deoxynucleotidyl transferase (TdT), as well as of a number of T antigens in mouse bone-marrow and spleen cells, and receptors for sheep erythrocytes in lymphocytes of immunodeficient humans (Goldschneider *et al.*, 1981; Goldstein *et al.*, 1981). Purification of TMS-F5 produces a group of peptides, of which thymosin α1, (TMS-α1) is chemically and biologically well characterized (Hu, Low & Goldstein, 1980; Ahmed *et al.*, 1979). TMS-α1 induces the differentiation of mouse bone-marrow T precursors at high concentrations, and of more mature T-cell subsets at low concentrations (Wetzel *et al.*, 1980; Goldstein *et al.*, 1981). Thymopoietin I and II have also been shown to induce the expression of thymic surface markers in murine marrow and spleen T

precursors and to induce TdT expression in a subset of mouse thymocytes (Trainin *et al.*, 1983). A pentapeptide (TP-5), corresponding to residues 32–36 of thymopoietin II, has been shown to retain the biological activity of thymopoietin II (Goldstein *et al.*, 1979).

Interleukin-2 (IL-2, or T cell-growth factor) is involved in the terminal differentiation of T cells (Bach & Papiernik, 1981; Ruscetti & Gallo, 1981). The non-physiological 12-O-tetradecanoyl phorbol acetate (TPA), a highly potent tumour promoter, has also been found to induce differentiation of leukaemic lymphoid cells *in vitro* (Nagasawa & Mak, 1980; Crossman *et al.*, 1982; Delia *et al.*, 1982).

Recently, it has been shown that different stages of T-cell differentiation are characterized by specific patterns of purine degradative enzymes adenosine deaminase (ADA), 5-ecto-nucleotidase (5'NT), purine nucleoside phosphorylase (PNP) and of TdT (Barton *et al.*, 1980; Ma *et al.*, 1982). During human T-cell maturation from cortical to medullary thymocytes there is a fall in TdT and ADA and a rise in PNP and 5'NT activities with the appearance of OKT3 surface antigen. Previous studies with thymic inducing agents have been with animal models or with human peripheral-blood T cells. We have now investigated whether the thymic factors (TMS-F5, TMS-α1, TP-5) and thymic epithelial culture conditioned medium (CM) and TPA have any effects on differentiation of normal human thymocytes *in vitro* by assessing changes in enzyme pattern and in surface phenotypic markers.

Since cloned leukaemic lymphoid cell lines are thought to represent different maturation stages in normal lymphoid development (Reinherz *et al.*, 1980; Greaves, 1979), we have also investigated the effect of the TMS-F5 and TPA on two thymic leukaemic lines.

MATERIALS AND METHODS

Thymocytes

Human thymic specimens were obtained from children undergoing open cardiac surgery (age range 3 months to 8 years old). The thymus specimens were cut into small fragments and thymocytes separated by gentle teasing. Cells were washed twice before suspension in RPMI-1640 supplemented with 2% heat-inactivated foetal calf serum (FCS), 2 mM L-glutamine and antibiotics. Cell viability was checked by trypan blue-dye exclusin and was always >95%. The cell count was performed by using a Coulter Counter Model S (Coulter Electronics, U.K). Since the percentage of cortical thymocytes was always over 80%, as reported previously (Ma *et al.*, 1982), no further purification was performed.

Cell lines

The cell lines RPMI-8402, JM1, (both derived from patients with Thy-ALL), KM3 (from patient with common acute lymphocytic leukaemia—cALL) and RAJI (from a B-cell Burkitt's lymphoma), were kindly donated by Dr M. F. Greaves, the Membrane Laboratories of the Imperial Cancer Research Fund, London, U.K. They were grown in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS), L-glutamine and antibiotics and gassed continuously with 5% CO_2 in humidified air. For the induction experiments, cells in logarithmic growth phase were used.

Reagents

TMS-F5 and TMS-α1 preparations were donated by Dr W. E. Scott (Hoffman La Roche, U.S.A). TP-5 was from Universal Biological Ltd. (Cambridge, U.K), IL-2 from Bethesda Research Laboratories Ltd, U.K., and TPA from Sigma Biochemicals, Poole, U.K. The monoclonal antibodies used were: OKT3, which characterizes medullary thymocytes and mature T lymphocytes, OKT11, which corresponds to receptors for sheep erythrocytes (from Ortho Diagnostics, U.K.) and NA1/34, which corresponds to OKT6 and characterizes immature T cells of the cortical thymocyte stage, (donated by Dr A. M. McMichael). The rabbit antibody to TdT was obtained from Bethesda Research Laboratories, Cambridge, U.K.

Thymocytes

The optimal conditions for the incubation of thymocytes with inducing reagents were estimated by chessboard titration with varying concentrations of the inducing agents and varying incubation times ranging from 11 to 48 hr. Changes in viability, percentage of OKT-positive cells and level of 5'NT were determined. The following optimal concentrations were found: TMS-F5, 300 μg/ml; TMS-α1, 0·2–0·4 μg/ml; TPA, 16 nM; CM, a 1:5 dilution. As no change in OKT3 or 5'NT could be found by using TP-5 and IL-2, they were used at concentrations reported to induce other changes, i.e. TP-5, 10 ng/ml (Nash *et al.*, 1981) and for IL-2, a 1:10 dilution (as recommended by the supplier BRL). An incubation time of 24 hr was found to be optimal, and the viability of the thymocytes at this

time was always more than 80% with no significant difference between controls and treated cells.

Thymic epithelial culture supernatant conditioned medium (CM)

The method was adopted from Gelfand, Dosch & Shore (1978). Pieces of human thymus were cut into small fragments and cultured in plastic petri dishes containing Alpha medium supplemented with 2 mM L-glutamine, 10% FCS and antibiotics. The medium was changed every 7 days. The cultures were maintained as described above for 14–21 days until thymic epithelial growth in monolayer could be found. The supernatant was collected and stored at 4°.

Induction experiments

Human thymocytes ($5–6 \times 10^6$/ml) in RPMI-1640 medium with 2% FCS were incubated with or without thymic factors or other substances for 18–72 hr at 37° in 5% humidified air with CO_2. The cells were washed, resuspended in phosphate-buffered saline (PBS), counted and divided into aliquots for enzyme and immunofluorescence tests. Cell viability was assessed both before and after incubation.

The leukaemic cell lines were incubated under similar conditions at a cell concentration of $1–2 \times 10^6$/ml. After 96 hr with or without TMS-F5 or TPA, the cells were washed, resuspended and aliquoted as above. This incubation time was chosen because previous studies showed that changes in differentiation markers induced by TMS and TPA were maximal after 72–96 hr (Ho *et al.*, 1983). Control and treated cells showed similar viability (> 95%) after incubation.

Immunofluorescence tests

The method has been described elsewhere (Ma *et al.*, 1982). The cells (1×10^6) were washed twice with and resuspended in PBS supplemented with albumin and sodium azide. The cells were incubated with the appropriate monoclonal antibodies (15 min, room temperature) and then with fluorescein isothiocyanate-conjugated goat anti-mouse antiserum. Cell suspensions were examined as wet preparations under a cover slip with a Zeiss photomicroscope II equipped with a fluorescent epi-illuminator and a 63-phase oil objective.

Enzyme assays

TdT activity was assayed as previously described (Hoffbrand *et al.*, 1977). All purine enzyme assays except for 5'NT were performed on cell extracts. The method have been described in detail elsewhere (Sylwestrowicz *et al.*, 1982). ADA was measured in a mixture containing [^{3}H]-adenosine (1 mM, 0·6 μCi) and 10 μl of cell extract (in 0·5 mM potassium phosphate buffer with 1 mM β-mercaproethanol pH 7·4) in a final volume of 50 μl. PNP was assayed by incubating 10 μl cell extract and inosine (2 mM 0·1 μCi) in phosphate buffer (25 mM pH 7·4) in a total volume of 100 μl for 40 min at 37°. 5'NT was assayed on intact cells; 1×10^6 cells in 59 μl of saline Tris buffer (0·04 M, pH 7·4) were incubated with equal volume of reaction mixture containing 2-[^{3}H]-adenosine-monophosphate (0·1 mM, 0·4 μCi) and magnesium chloride (3 mM) for 15 min at 37°.

Statistical analysis

Tests for statistical differences of the means were performed according to Student's *t* test for unpaired samples.

RESULTS

Normal thymocytes

The changes in enzyme levels of TdT, ADA, PNP, 5'NT in the percentage of cells positive for TdT, OKT3 and NA1/34 are summarized in Table 1. No change in enzyme levels or antigenic markers occurred in control thymocytes within 24–36 hours of incubation.

In thymocytes treated with TMS-F5 there was a significant increase in cells positive for OKT3 from $34·7 \pm 6·0$ to $52·1 \pm 18·9$% ($P < 0·01$) and a significant reduction of cells positive for TdT from $75·4 \pm 8·6$ to $63·9 \pm 12·7$% ($P < 0·05$).

TMS-α1 significantly decreased the biochemical levels of TdT ($P < 0·01$), cells positive for TdT ($P < 0·01$) and the level of ADA ($P < 0·05$). There was a significant increase of cells positive for OKT3 ($P < 0·01$). On the other hand, the only significant change caused by TP-5 was an increase in PNP level ($P < 0·01$), and by CM a rise in 5'NT level ($P < 0·05$).

In the cells treated with TPA, a reduction of both TdT enzyme activity and the percentage of cells positive for TdT ($P < 0·001$) occurred with a significant increase of PNP ($P < 0·01$). However, TPA also caused a significant decrease of cells positive for OKT3 ($P < 0·001$).

Human leukaemic lymphoid cell lines

TMS-F5 caused a significant increase in 5'NT levels in

Table 1. Effects of the thymic and other factors on enzymes and antigenic markers of normal human thymocytes

	TdT (pmol/hr/ 10^8 cells)	ADA (μmol/hr/ 10^8 cells)	PNP (nmol/hr/ 10^6 cells	5′NT (nmol/hr/ 10^6 cells)	TdT-IF (%)	OKT3 (%)	NA1/34 (%)
Control	5·7±1·9	39·0±18·6	27·5±8·5	1·5±0·6	75·4±8·6	34·7±6·0	80·8±6·9
TMS-F5	6·3±2·4	27·7±15·9	31·5±12·4	1·4±0·9	63·9±12·7*	52·1±18·9**	81·7±5·1
TMS-α1	3·6±1·6**	21·0±12·8*	26·0±7·4	1·9±0·7	60·2±8·5**	42·5±9·8**	71·9±8·6
TP-5	3·8±2·2	32·8±15·2	38·1±5·7**	1·2±0·3	68·7±13·1	43·7±22·0	—
CM	5·2±1·2	33·2±16·4	27·3±2·9	2·4±1·1*	78·7±11·0	40·0±7·1	87·0±1·4
IL-2	5·4±2·7	42·0±24·4	28·1±9·3	1·5±0·8	74·5±8·1	36·7±8·0	82·9±4·9
TPA	0·7±0·4***	54·2±24·8	41·5±11·5**	1·5±0·7	34·3±10·9***	22·0±6·6***	78·7±5·8

Abbreviations as in text.
Results are given as mean±SD of five to 15 experiments.
Significant changes compared to controls are shown by asterisks (*$P<0·05$; **$P<0·01$; ***$P<0·001$).

both the T-cell lines: RPMI-8402, which has an 'early' thymic phenotype ($P<0·01$) and JMI ($P<0·05$), which has a 'cortical' thymic phenotype (Delia *et al.*, 1982) (Table 2). In KM3 (derived from cALL) and RAJI (derived from B-ALL), TMS-F5 did not result in any enzyme or phenotypic marker changes.

TPA, on the other hand, caused changes in all the cell lines. In RPMI-8402, it reduced TdT assessed biochemically or immunologically, but also reduced the 5′NT level ($P<0·001$). In JM1 it caused a reduction in the TdT-positive cells ($P<0·001$) and a reduction of the antigenic markers NA1/34 and OKT3 ($P<0·001$ in both cases). In KM3, TPA caused highly significant reduction of both TdT and ADA ($P<0·001$) activities with a marked elevation of 5′NT level ($P<0·001$). In the B-derived line, RAJI, TPA increased the levels of both ADA and PNP ($P<0·05$).

DISCUSSION

Previous studies have shown that during normal differentiation of cortical thymocytes into mature T cells, there is a fall in the levels of TdT and ADA, and

Table 2. Effects of the various factors on enzyme and immunological markers in lymphoid leukaemic cell lines: RPMI-8402, JM1, (both Thy-ALL lines), KM3 (cALL line) and RAJI (B-ALL line)

		TdT (pmol/hr/ 10^8 cells)	ADA (mol/hr/ 10^8 cells)	PNP (nmol/hr/ 10^6 cells)	5NT (nmol/hr/ 10^6 cells)	TdT-IF (%)	OKT3 (%)	NA1/34 (%)
RPMI-8402	Control	6·3±0·9	261±25·0	220·6±62·0	9·8±1·5	91·3±0·6	0	0
	TMS-F5	8·5±0·2	227·0±21·2	225·0±41·5	27·3±7·6**	88·3±7·2	0	0
	TPA	1·7±0·9***	192·0±63·0	266·7±35·1	3·8±1·1**	31·3±21·4***	0	0
JM1	Control	0·2±0·1	172·2±24·1	198·6±13·7	3·8±1·5	79·3±15·0	36·6±13·3	65·3±9·4
	TMS-F5	0·4±0·1	162·1±17·2	207·3±13·2	9·6±4·5*	61·3±24·6	36·7±4·5	58·4±14·8
	TPA	0·3±0·2	168·0±40·4	191·2±27·7	4·3±0·9	39·3±11·0***	16·0±4·2***	21·3±3·8***
KM3	Control	118·9±36·5	33·3±10·0	137·6±13·4	4·4±1·5	87·0±1·4	0	0
	TMS-F5	126·2±32·6	34·7±6·6	159·1±12·6	5·4±1·6	80·5±7·8	0	0
	TPA	4·9±2·0***	9·6±2·1***	108·3±22·3	19·2±4·2***	28·5±0·7***	0	0
RAJI	Control	1·2±0·5	14·6±1·8	365·5±16·8	<0·1	0	0	0
	TMS F-5	1·0±0·1	15·2±4·4	365·9±22·5	<0·1	0	0	0
	TPA	0·7±0·1	30·3±7·1*	454·1±14·8*	<0·1	0	0	0

Abbreviations as in text.
Results are given as mean±SD of three to five experiments.
Significant changes compared to control are shown by asterisks (*$P<0·05$; **$P<0·01$; ***$P<0·001$).

inversely a rise in the activities of PNP and 5′NT (Barton *et al.*, 1980; Ma *et al.*, 1982). Moreover, thymocytes lose the surface antigenic marker NA1/34 (or OKT6) and acquire OKT3 (Tidman *et al.*, 1981). All the thymic factors studied here caused one or more of these changes in human thymocytes, with TMS-F5 a reduction of cells positive for TdT and increase in OKT3-positive cells, with TMS-α1, a fall in TdT and ADA, and an increase in cells positive for OKT3. Other authors have demonstrated that TMS-α1 is able to decrease TdT expression in murine thymocyte *in vitro* and have suggested that TMS-α1 acts at late stages of thymocyte maturation (Goldstein *et al.*, 1981; Hu *et al.*, 1980; Wetzel *et al.*, 1980). Our results on purine enzymes, TdT and phenotypic changes confirm this concept in human thymocytes. It was, however, obvious that TMS-α1 did not induce all the changes which characterize differentiation of cortical thymocytes. There was no change in PNP, 5′NT and NA1/34. An increase in PNP activity was brought about by TP-5, however, and an elevation in 5′NT activity by CM. TP-5 induces early T-cell differentiation in murine cells (Goldstein *et al.*, 1979). Cohen, Dosch & Gelfand (1981) have demonstrated a rise in 5′NT levels in human thymocytes incubated with thymic conditioned media or with TMS-F5. We are able to confirm their observation with conditioned media but not with TMS-F5.

Our results with the thymic factors, TMS-F5, TMS-α1, TP-5 and CM thus suggest that these physiological factors might supplement each other, the maturation of cortical thymocytes proceeding stepwise, each step requiring one or more thymic hormones.

The changes in TdT and purine degradation enzymes induced by the thymic factors in the normal human thymocytes were less marked than in the changes that occur during normal differentiation from cortical to medullary thymocytes and to mature T cells (Ma *et al.*, 1982). This difference might be partly due to heterogeneity of the thymocytes. Previous studies have shown that 80% of unseparated thymocytes are cortical thymocytes, 15% medullary thymocytes and 1–5% prothymocytes. Another possibility is that only a proportion of the cortical thymocytes are able to differentiate into mature T cells. In fact, it is known that *in vivo*, most cortical thymocytes die within the thymus (Scollay, Butcher & Weisman, 1980).

It has recently been suggested that the particular enzyme pattern in each developmental stage of T cells might be essential for the cell survival and might account for the 'biochemical suicide' of cortical thymocytes (Ma *et al.*, 1983). It is known that thymocytes are prone to deoxyribonucleotide accumulation. The high TdT and ADA activity in cortical thymocytes might serve to protect these cells but at the late cortical thymocyte stage, the fall in TdT with the low PNP and 5′NT activities creates a suicidal combination due to toxic accumulation of purine nucleotides. Thymocytes with the ability of self recognition might survive by rapid induction of differentiation to a stage where the cells contain high levels of PNP and 5′NT, i.e. an efficient purine degradation capacity. We have shown in the present study that thymic hormones do induce such enzyme changes in human cortical thymocytes. A tentative suggestion is that TMS-α1 is needed in a first step loss of TdT and ADA, and that other thymic hormones, e.g. TP-5, and factors present in CM, might be required to induce or augment the activities of PNP and 5′NT.

Thymosin (TMS-F5) caused a marked elevation of 5′NT in two malignant T-cell lines, RPMI-8402, which has an 'early' phenotype according to surface marker analysis, and in JM1, which has a phenotype equivalent to cortical thymocytes (Delia *et al.*, 1982). On the other hand, TMS-F5 did not cause such changes in KM3 (derived from cALL) and in RAJI (derived from Burkitt's lymphoma). This supports the concept that the action of thymosin is specific on T cells. It also is consistent with our previous study (Ho *et al.*, 1983) showing that TMS-F5 could induce part of the changes of normal differentiation in malignant T cells. It is of interest, however, that different patterns of changes are induced by the same reagent in leukaemic cells and in their normal counterpart, observations which have already been made for myeloid leukaemia (Sachs, 1982).

Finally, the non-physiological compound TPA also caused a number of changes which could be interpreted as equivalent to differentiation. However, changes were also induced that were not compatible with maturation, e.g. reduction of OKT3 expression in normal thymocytes and in JM1, decrease in 5′NT levels in RPMI-8402 and JM1. These changes are more consistent with simultaneous de-differentiation in these cells. The action of TPA is also not specific, since changes were also induced in cALL and B lymphoblastic lines. It is, in fact, unclear whether the changes caused by TPA represent maturation at all.

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