

INDUCTION IN VIVO AND IN VITRO OF TERMINAL
DEOXYNUCLEOTIDYL TRANSFERASE BY
THYMOSIN IN BONE MARROW CELLS FROM ATHYMIC
MICE*

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Terminal deoxynucleotidyl transferase (TdT)¹ is currently thought to be a T-cell-specific enzyme. The enzyme was initially purified from calf thymus (1) and it catalyzes the polymerization of deoxynucleotides in the presence of a primer (1-3). Despite extensive research of the in vitro catalytic properties of the enzyme (4, 5), the in vivo function of the enzyme is unknown. In a variety of species that have been examined (6-8), TdT is only found in thymocytes or bone marrow cells, suggesting a specific T-cell association. In rats, TdT activity appears to be restricted to the major thymocyte subpopulation, which is characterized by small size, cortisone sensitivity, and moderate density (7, 9). Based on these data, it has been suggested that TdT functions in the early stages of T-cell development (9). TdT is also found in the peripheral sites of leukemic cell infiltration in T-cell leukemias of several species (10-16). These data have suggested that the expression of TdT may be a characteristic of T-cell leukemias and may reflect a block in differentiation (7).

Although considerable research has dealt with the distribution of TdT in T-cell subpopulations, little is known of the molecular mechanisms controlling either TdT expression or thymocyte differentiation. In murine thymocytes two TdT activities are separable by phosphocellulose chromatography (peak I and peak II), which have comparable in vitro primer and substrate requirements, and which appear to be serologically identical (8, 16, 17). However, the two enzyme activities have very distinct biological properties in that in certain strains of mice (NIH Swiss and AKR), peak II activity is rapidly lost with age, whereas peak II activity in other strains (C57BL/6) does not decline until at least 6 mo of age. In all strains examined, peak I activity is comparable and retained with age (16). More interestingly, however, peak I in all strains examined is uniquely associated with leukemic T cells infiltrating peripheral

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¹ *Abbreviations used in this paper:* BSA, bovine serum albumin; dGTP, deoxyguanosine 5'-triphosphate; TdT, terminal deoxynucleotidyl transferase; TEM, a buffer of 0.05 M Tris, pH 7.8, 1 mM of EDTA, and 1 mM of 2-mercaptoethanol; TEM-G, TEM plus 20% glycerol.

lymphoid tissues (16). In mice, TdT is also thought to be associated with a prothymocyte population in the bone marrow. This hypothesis was suggested by the observation that TdT-containing bone marrow cells could be lysed with antisera against theta after treatment *in vitro* with thymopoietin (18). These experiments, however, were compromised by the extremely low activity of TdT normally detectable in whole bone marrow. More recently, it has been shown that TdT is associated with a unique bone marrow subpopulation separable by bovine serum albumin (BSA) gradient centrifugation (19). The enzyme-specific activity of TdT in this subpopulation, which comprises only 1–5% of the total bone marrow cells, is comparable to that of thymocytes. Interestingly, only low levels of TdT were present in this bone marrow fraction of NIH Swiss nude mice, suggesting that TdT expression in bone marrow is possibly regulated by thymic factors. In the present experiments, we have extended these observations and examined the ability of thymic hormones to induce TdT in athymic mice. The results demonstrate that thymosin fraction 5 can induce the *de novo* appearance of TdT *in vitro* in specific bone marrow subpopulations of athymic mice.

Materials and Methods

Mice. C57BL/6 and NIH Swiss nu/nu specific pathogen-free mice used in these experiments were obtained from the Frederick Cancer Research Center Animal Breeding Section, Frederick, Md. Ages of the different mice are given in the Results section.

Cell Fractionation. Preparation of cell suspensions from spleens, lymph nodes, and bone marrows has been previously described (16). The method of Raidt et al. (20) was used to fractionate the different cell suspensions. Four layers of cells were formed at the interfaces of the discontinuous BSA gradient: fraction A, between 10 and 23% BSA; fraction B, between 23 and 26%; fraction C, between 26 and 29%; and fraction D, between 29 and 33% BSA. BSA (Path-O-Cyte 5, lots 25 and 26) was obtained from Miles Laboratories Inc., Miles Research Products (Elkhart, Ind.). Fractionated cells were washed three times in Ham's F-12 medium.

Enzyme Preparation. Approximately 1×10^6 cells from the different layers were resuspended in a 1-ml buffer solution of 0.05 M Tris, pH 7.8, 1 mM EDTA, and 1 mM 2-mercaptoethanol (TEM), and frozen-thawed. Immediately after thawing, 3 ml of TEM buffer containing 2 M KCl and 0.66% Triton were added. The homogenate was stirred at 4°C for 2–4 h and dialyzed overnight against 100 vol of TEM buffer containing 50 mM KCl.

The dialyzed solution was centrifuged at 100,000 *g* for 60 min, and the supernate was loaded on a 1×15 -cm P11 phosphocellulose column (Whatman, Inc., Clifton, N. J.) and equilibrated with 50 mM KCl in TEM plus 20% glycerol (TEM-G). After loading, the column was washed with 15 ml of TEM-G, 50 mM KCl, and then eluted with a linear gradient (50 ml) of 0.05–0.75 M KCl in TEM-G buffer. The flow rate of the column was 1–1.2 ml/10 min, and 1.0-ml fractions were collected.

TdT Assay. The TdT assay was adapted from Kung et al. (8) and has been previously described. One unit of enzyme activity was defined as the amount catalyzing the incorporation of 1 pmol of deoxyguanosine 5'-triphosphate (dGTP) into acid-insoluble material per h. Specific activity was calculated from the total enzyme activity recovered from phosphocellulose per 10^6 nucleated viable cells. Under the conditions of the assay, the lowest detectable activity is ≈ 0.05 pmol of dGTP incorporated.

Thymectomy. 3-wk-old C57BL/6 mice were thymectomized using the procedure of Sjodin et al. (21).

Thymosin Treatment In Vivo. Thymosin fraction 5 (lot BPM 390), and spleen fraction 5 (lot 307), purified as previously described (22, 23), were resuspended in saline solution at a concentration of 500 μ g/ml.

10 daily injections of 100 μ g were given intraperitoneally to 6-wk-old NIH Swiss nu/nu or to 15-wk-old C57BL/6 mice, which had been thymectomized at 3 wk of age. Animals were sacrificed 24 h after the last injection.

For the in vitro induction experiments, cells were washed three times after BSA gradient fractionation in Ham's F-12 medium containing 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10 μ g/ml gentamycin. After washing, cell concentrations were adjusted to 1×10^8 cells/ml in the same medium. The different fractions were plated at 1×10^8 cells/dish in 10 ml of Ham's F-12 medium containing antibiotics, 5% fetal bovine serum (Flow Laboratories, Inc., Rockville, Md.), and different concentrations of thymosin or spleen fraction 5. After incubation for various periods of time, cells were collected, washed twice with Ham's F-12, and extracted as previously described for TdT activity (16).

Actinomycin-D Treatment and [³H]Uridine Labeling. Actinomycin D, at 0.5 μ g/ml, was incubated for 12 h in the presence of thymosin and fraction B from the bone marrow of NIH Swiss nu/nu mice to study their effect on TdT induction. To follow the effect of actinomycin D on RNA synthesis, cells were labeled with 10 μ Ci/ml of [³H]uridine for the last 90 min of incubation.

Results

The distribution of TdT in BSA gradient-fractionated bone marrow cells from various strains of mice is shown in Table I. As previously reported (19), and as is illustrated here by the results with NIH Swiss and C57BL/6 mice, TdT is primarily associated with cells of the A fraction. The fact that the peak II TdT activity is lost early in life in NIH Swiss mice is also consistent with previous results (16) that there is a difference in enzyme expression between these two strains. Also shown in Table I are the results obtained with BSA gradient-fractionated lymphoid tissues of NIH Swiss nude mice. These mice had only low levels of TdT in the bone marrow fraction A cells and no TdT activity was detectable in gradient-fractionated cells of either spleen or lymph nodes. These results suggest that either the thymus or thymic factors are required for TdT expression in the bone marrow.

To examine further the influence of the thymus on TdT expression in the bone marrow, 3-wk-old C57BL/6 mice were thymectomized and TdT expression in bone marrow was followed with time after thymectomy. As is illustrated in Table II, thymectomy had a significant influence on TdT activity in the bone marrow. By 8 wk after thymectomy, >95% of the peak II activity was lost and only \cong 35% of the peak I activity remained. By 16 wk after thymectomy, >95% of the total TdT activity was lost. At no time after thymectomy was enzyme activity detected in either spleen or lymph nodes (data not shown). These results are therefore consistent with the results from nude mice in suggesting a role for the thymus in the regulation of TdT in the bone marrow.

It has been previously demonstrated that T-cell functions can be enhanced by the administration of thymus extracts (24-27). We therefore examined the ability of thymosin fraction 5, a partially purified thymus extract, to restore TdT expression in bone marrow of thymectomized and nude mice. As is shown in Table III, 10 daily injections of 100 μ g of thymosin fraction 5 significantly increased TdT activity in bone marrow fraction A cells of both nude and thymectomized mice, which is consistent with the normal cellular distribution of TdT activity. The effect was specific for thymosin fraction 5 in that neither spleen fraction 5 nor saline had significant effects. Interestingly, thymosin treatment of nude mice resulted primarily in the appearance of peak I activity at a specific activity comparable to nu/+ littermates and little peak II activity, in a manner consistent with the strain-dependent differences normally seen in the two activities (16). However, both TdT peaks I and II were induced in

TABLE I
Distribution of Terminal Transferase Activity in BSA-Fractionated Lymphoid Tissues from Normal and Athymic Mice

	BSA fraction							
	A		B		C		D	
	I	II	I	II	I	II	I	II
NIH Swiss nu/+ bone marrow	145*	-‡	6	-	1	-	-	-
C57BL/6 bone marrow	225	1,364	15	180	-	8	-	15
NIH Swiss nu/nu bone marrow	16	-	3	-	1	-	-	-
NIH Swiss nu/nu spleen	-	-	-	-	-	-	-	-
NIH Swiss nu/nu lymph nodes	-	-	-	-	-	-	-	-

* Picomoles of [³H]dGTP incorporated per h per 10⁸ cells. Mice used were 6 wk of age.

‡ Not detectable.

TABLE II
TdT Activity in Thymectomized C57BL/6 Mice*

Weeks after thymectomy	Bone marrow fractions							
	A		B		C		D	
	I	II	I	II	I	II	I	II
0	195‡	1,310	41	110	2	6	-§	-
1	240	360	70	130	5	5	7	10
8	71	-	15	5	3	-	-	-
16	11	-	-	-	-	-	-	-

* C57BL/6 mice thymectomized at 3 wk of age and assayed at various times after thymectomy.

‡ Picomoles of [³H]dGTP incorporated per h per 10⁸ cells.

§ Not detectable.

thymectomized C57BL/6 mice at specific activities comparable to those seen in normal thymic-bearing controls. No TdT activity was detectable in fractionated cells from either spleen or lymph nodes of thymosin-treated mice (data not shown).

The results of in vivo thymosin treatment suggested that thymosin fraction 5 can promote TdT expression in bone marrow. However, it was not possible to differentiate between a direct inductive event or a secondary effect on differentiation. We therefore examined the ability of thymosin fraction 5 to induce TdT in vitro in fractionated bone marrow and spleen cells from NIH Swiss nude or thymectomized C57BL/6 mice. The results obtained with NIH Swiss nude mice are shown in Table IV. When fractionated spleen or bone marrow cells were treated with 25 ng/ml of thymosin fraction 5 for 18 h, TdT was specifically

TABLE III
*In Vivo Reconstitution of TdT Activity in Bone Marrow Cells from Nude and Thymectomized Mice with Thymosin Fraction 5**

	Bone marrow fractionated on BSA															
	NIH Swiss Nu/Nu								Thymectomized C57BL/6							
	A		B		C		D		A		B		C		D	
	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
Thymosin fraction 5	210	3	32	-‡	3	-	2	-	141	916	24	69	5	10	-	-
Spleen fraction 5	18	-	10	-	3	-	-	-	21	136	2	7	5	3	3	6
Saline	22	4	9	1	-	-	-	-	12	2	4	-	-	-	-	-

* NIH Swiss nude and thymectomized C57BL/6 were given 10 daily injections of spleen fraction 5, thymosin fraction 5, or saline, i.p.; 24 h after the last injection, the mice were sacrificed and TdT was isolated as described in Materials and Methods.

‡ Not detectable.

TABLE IV
*In Vitro Induction of TdT in Fractionated Spleen and Bone Marrow Cells from NIH Swiss nu/nu Mice by Thymosin Treatment**

Treatment		BSA fraction			
		A	B	C	D
Saline	Spleen	-‡	-	-	-
	Bone marrow	2.5	0.9	-	1.0
Spleen fraction 5	Spleen	-	-	-	-
	Bone marrow	13.4	-	-	-
Thymosin fraction 5	Spleen	8.1	-	-	-
	Bone marrow	2.0	131	-	-

* Values for peak I activity only. Incubation was for 18 h with 75 ng/ml of thymosin or spleen fraction 5.

‡ Not detectable.

induced in fraction B bone marrow cells. Moreover, the specific activity of the enzyme was $\cong 60\%$ of the value found after in vivo thymosin treatment, and it had the characteristics of peak I activity. The in vitro induction was specific in that neither spleen fraction 5 nor saline significantly increased enzyme activity, and activity was not induced in either fractionated spleen cells or other fractions of bone marrow cells. Comparable results were obtained for the in vitro induction of TdT in bone marrow cells from thymectomized C57BL/6 mice (Table V). As above, thymosin fraction 5 specifically induced TdT in the B-fraction cells. In contrast to the results with NIH Swiss nude mice, however, both peaks I and II were induced at ratios comparable to those normally found, and at a specific activity of $\cong 50\%$ of the normal bone marrow fraction A cells. Spleen fraction 5 had only a minimal inductive effect and yielded $\cong 10\%$ of the specific activity found with thymosin fraction 5. These results demonstrate, therefore, that thymosin fraction 5 can directly induce TdT in vitro in bone marrow cells from athymic mice.

TABLE V
In Vitro Induction of TdT in Fractionated Spleen and Bone Marrow Cells from
 Thymectomized C57BL/6 Mice by Thymosin Treatment

	BSA fraction							
	Bone marrow				Spleen			
	A		B		A		B	
	I	II	I	II	I	II	I	II
Saline	19	—*	8	—	—	—	—	—
Spleen frac- tion 5	25	4	35	71	—	—	—	—
Thymosin fraction 5	21	10	91	673	—	—	—	—

* Not detectable.

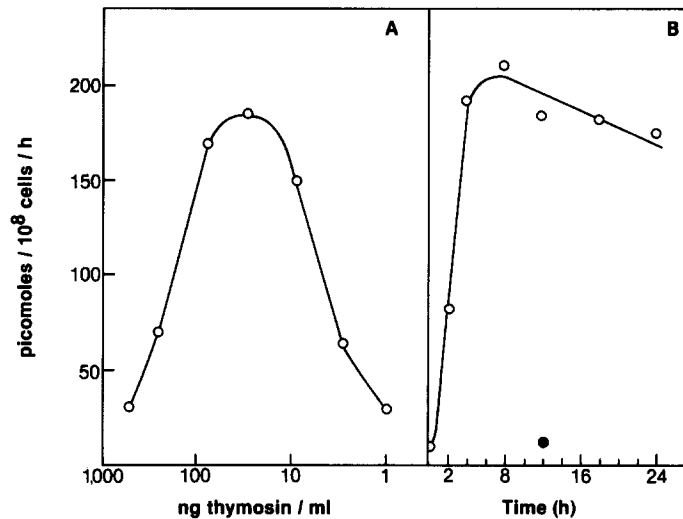


FIG. 1. Concentration and time dependence of the induction of TdT by thymosin fraction 5. NIH Swiss nu/nu bone marrow cells were fractionated on BSA gradients and the B layer was used in induction experiments. (A) The cells were treated with varying concentrations of thymosin fraction 5 for 12 h and then extracted; TdT activity was determined after phosphocellulose chromatography. (B) Cell suspensions were treated with 25 ng/ml of thymosin fraction 5 in the presence (●) or absence (○) of 0.5 μ g/ml of actinomycin D. At the indicated intervals, samples of the cells were collected, and extracted; TdT activity was determined after phosphocellulose chromatography. Only TdT peak I was detected in these samples.

The concentration dependence for the induction of TdT in fraction B, nude bone marrow cells by thymosin fraction 5 is shown in Fig. 1 A. Concentrations as low as 3 ng/ml had a significant effect, and \cong 30 ng/ml was optimal. At higher concentrations, there was a significant inhibitory effect which was not associated with cell death (data not shown). The kinetics of induction using 25 ng/ml are shown in Fig. 1 B. As illustrated by the results, induction *in vitro* is rapid, and within 2 h a significant increase in TdT is evident. By 4–6 h the cells are fully induced, and enzyme activity remains constant for up to 24 h. Also

illustrated in Fig. 1 B are the results obtained at 12 h in the presence of 0.5 $\mu\text{g}/\text{ml}$ of actinomycin D. Under these conditions, >95% of the RNA synthesis is inhibited (data not shown), as is the induction of TdT. Taken together, these results suggest that thymosin fraction 5 induction of TdT is comparable to the pattern of induction observed for other enzymes in response to specific hormones.

Discussion

The results clearly demonstrate that TdT expression in the bone marrow is dependent upon the presence of a thymus, and that TdT can be induced in the bone marrow of athymic mice both *in vivo* and *in vitro* after treatment with thymosin fraction 5. An important role for the thymus in TdT expression had been previously suggested based on the observation that NIH Swiss nude mice had only low levels of TdT in fractionated bone marrow cells (19). We tested this hypothesis further by examining fractionated bone marrow from thymectomized mice for TdT expression. As the results illustrated, thymectomy had a profound effect on TdT expression in the bone marrow of C57BL/6 mice, so that by 16 wk after thymectomy, <5% of the initial activity was detectable. Interestingly, there was a differential rate of loss of peak I and peak II activities, recalling the loss of activity seen during aging (16). In particular, peak II was lost rapidly after thymectomy, whereas peak I was lost at a much slower rate. This observation, in addition to the other age, tumor, and strain differences previously noted (16, 19), suggests that peak I and peak II activities are biologically different and may have distinct functional properties. However, whether the two peaks of activity are unique enzymes or one enzyme with two forms is not known, since the data are also consistent with a peak II to peak I conversion.

Treatment of either NIH Swiss nude mice or thymectomized C57BL/6 mice with thymosin fraction 5 induced, *in vivo*, the expression of TdT in fractionated bone marrow cells. This induction was specific to thymosin fraction 5 in that a hormone preparation comparable to thymosin in its preparation but isolated from calf spleen had no effect, nor did saline. The induction of TdT *in vivo* was striking since the specific activity of the enzyme was comparable to that seen in normal, thymic-bearing controls and since the enzyme was primarily associated with the A fraction of BSA-gradient fractionated cells. In addition, the ratios of peak I and peak II were identical to the thymic-bearing controls, and they illustrated the strain-dependent differences normally observed. These data suggest that the differences seen in peak I and II expression are probably not related to differences in the factors required for induction, but are rather at the cellular level of enzyme expression as discussed below.

Although thymosin fraction 5 clearly induced TdT expression *in vivo*, such experiments can not distinguish between a direct inductive event or a promotion of differentiation, which has as a secondary consequence the expression of TdT. We therefore examined the ability of thymosin fraction 5 to induce TdT *in vitro* in fractionated bone marrow cells from athymic mice. The results clearly demonstrated that thymosin fraction 5 can induce enzyme activity *in vitro* very effectively, suggesting a direct hormone-like effect on TdT expression. As above, the effect was specific in that neither spleen fraction 5 nor saline had a

comparable effect. In contrast to the above results, however, TdT was primarily induced in the B fraction of cells from BSA gradients, whereas *in vivo* TdT is primarily associated with the A fraction. These data might be best interpreted to suggest that the B fraction contains the stem cell for the TdT-positive A-fraction cells. Experiments are currently in progress to examine this question by determining the density distribution of TdT-expressing cells after *in vitro* induction. Previous reports have demonstrated that fraction B can effect thymic reconstitution of irradiated mice, although the A fraction also had more limited capabilities (28). Whether or not the ability to express TdT and to reconstitute the thymus resides in the same stem-cell populations is not known. However, the observation that the B-fraction cells from spleens can effect thymic reconstitution, but as shown here, are not inducible for TdT, suggests that these two functions may belong to different cell populations.

In a manner similar to the results *in vivo*, induction of TdT *in vitro* with thymosin fraction 5 resulted in only peak I activity in cells from NIH Swiss nude mice, and peaks I and II in cells from thymectomized C57BL/6 mice. These results clearly demonstrate that the difference between peaks I and II is a strain-dependent difference in the cellular response to thymic factors, rather than a difference in the requisite hormones. In particular, it was conceivable that peak I and peak II were regulated by different hormones and that in certain strains, the hormone-inducing peak II is lost early. As is shown here, however, a thymic hormone preparation capable of inducing peak II activity in C57BL/6 bone marrow cells did not induce peak II activity in NIH Swiss nude bone marrow. Thus, it is still unknown what cellular factors are involved in the differences observed here.

The *in vitro* induction of TdT demonstrated here appears to be comparable to many classical examples of hormone induction of enzymes. The concentrations of thymosin required are extremely low (25 ng/ml) and well within physiological levels. Comparably low levels of thymosin have been shown to induce theta antigen expression (26). It should also be noted that since thymosin fraction 5 contains several peptides (29), the actual concentration of the active fraction, assuming a single peptide is involved, may be below 1 ng/ml. The concentration dependence for induction shows a marked inhibition at high thymosin concentrations. This effect does not appear to be the result of cell killing, but it may be related to the ability of thymosin to promote a variety of thymic functions (22, 23, 25-27, 29). Thus, if several hormonally active peptides are present in thymosin, competition may exist in promoting differentiation. For example, in preliminary experiments we have examined the effect of thymosin alpha-1 on the induction of TdT *in vitro*, and we found that it does not induce the enzyme. Thymosin alpha-1, which is the first thymic peptide hormone sequenced, has a mol wt of 3,100 and is 10-100 times as active as fraction 5 in a number of thymosin assays (29). In this regard, it should also be noted that thymosin fraction 5 can induce expression of theta in nude mice, and it now becomes imperative to know whether the same or different thymic factors are involved in TdT and theta expression. Clearly, these phenomena can only be resolved by examining the effects of purified peptides from thymosin.

The time course for induction of TdT expression *in vitro* is extremely rapid

and comparable to the patterns observed in vitro with other enzymes and hormones. Interestingly, induction is complete within 6 h, and the level of enzyme activity is maintained relatively constant for up to 24 h. Whether or not the maintenance of enzyme activity requires the continued presence of thymosin fraction 5 is presently under investigation. The results also demonstrate that the induction of TdT is completely inhibited by actinomycin D, suggesting that RNA synthesis is required, and that the expression of TdT requires de novo synthesis of the enzyme. Clearly, however, continued efforts will be required to establish this point definitively. Nevertheless, our results demonstrate that a unique T-cell enzyme can be induced in vitro with thymic hormones.

Summary

Terminal deoxynucleotidyl transferase (TdT) expression in bovine serum albumin (BSA) gradient-fractionated bone marrow cells was examined in NIH Swiss nu/nu and thymectomized C57BL/6 mice. In nude mice, TdT levels were approximately 10% of those of thymus-bearing littermates. In C57BL/6 mice, thymectomy caused a time-dependent loss of TdT activity in bone marrow cells. To determine whether or not the apparent thymic requirement for TdT expression in bone marrow was mediated by thymic hormones, we examined the effects of thymosin fraction 5. Treatment of either NIH Swiss nu/nu or thymectomized C57BL/6 mice with thymosin fraction 5 restored the levels of TdT activity in BSA gradient-fractionated bone marrow cells to normal. Moreover, treatment of BSA gradient-fractionated bone marrow cells from NIH Swiss nu/nu or thymectomized C57BL/6 mice in tissue culture with thymosin fraction 5 induced TdT expression. In tissue culture, TdT induction was optimal with 25 ng/ml of thymosin fraction 5, it occurred within 6 h, and it was completely inhibited by actinomycin D. The effect was specific in that neither control nor spleen fraction 5-treated cells were induced to express TdT. These data demonstrate that TdT expression in bone marrow cells is under the direct control of thymic polypeptide hormones.

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