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TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE ACTIVITY IN THYMUS BIOPSIES OF IMMUNODEFICIENT PATIENTS

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Terminal deoxynucleotidyl transferase (TdT) is a DNA polymerase which in vitro adds deoxynucleotides to the 3′ OH end of a primer without a template. This function and its distribution in bone marrow pre-T cells and thymocytes suggest a role for this enzyme in the development of the immune system. We measured TdT activity in thymus biopsy specimens of patients with immunodeficiency disease to gain possible insight into the biology of this enzyme and/or the pathogenesis of immune deficient states. Thymuses from four patients with severe combined immunodeficiency disease (SCID) and one with DiGeorge syndrome were assayed for enzyme activity with and without ATP, a specific inhibitor of TdT. In two patients with SCID, specific enzyme activity (pmol/0.5 g tissue) was 50 and 157%, respectively, of age and sex-matched controls; one patient had no enzyme activity. TdT activity in the only adenosine deaminase deficient patient was 23% and was not inhibited by ATP. Crude extract from the DiGeorge thymus had 42% activity, but was not tested with ATP. Purification of crude extracts by DEAE, phosphocellulose and oligodT chromatography was performed to determine if specific enzyme activity had been masked. After phosphocellulose chromatography, ATP noninhibitable activity was removed from one fraction pool in one patient, but not from a second pool. Two other patients had TdT activity in low salt eluates (0.05 M KCl) rather than where expected. Purification of these samples by oligodT chromatography resulted in specific enzyme activity comparable to age-matched controls. The known inhibition of TdT activity by ATP suggests that the nucleotides and deoxynucleotides which accumulate in lymphocytes in SCID may account for the abnormal TdT activity seen in these patient specimens. Examination of TdT activity in immunodeficient patients may allow delineation of this enzyme's biological function.

Keywords: immunodeficiency; terminal transferase; thymus

INTRODUCTION

The classification, diagnosis and treatment of immunodeficiency diseases is conventionally based on the phenotypic appearance of the patient's clinical features or laboratory abnormalities. Stem cell defects have been cited as the etiology of combined defects [1], whereas specific organ failure [2,3] or imbalances among

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regulator cells [4] have been proposed as the causes for more select deficiencies. Only recently have defects in enzymes or carrier proteins (adenosine deaminase [5], nucleoside phosphorylase [6], 5′ nucleotidase [7], carboxylase [8], and transcobalamin 11 [9]) been identified as the cause of immune deficiencies. The determination of other specific deficiencies is desirable so that a further understanding of immune function and specific therapy may be developed.

Terminal deoxynucleotidyl transferase (TdT), an enzyme normally found in thymus and bone marrow of vertebrates [10,11], is a DNA polymerase which in vitro adds deoxynucleotides to the 3′ OH end of a primer in the absence of a template [12]. The in vivo function of this enzyme is unknown although it has been linked with the development of immune diversity [13] and/or maturation of T-lymphocyte function [14]. It, therefore, was of interest to measure TdT in thymus of patients with defects in immunologic development. The results reported here indicate that TdT is present in the thymus of patients with severe combined immunodeficiency or the DiGeorge syndrome, but its expression is abnormal.

MATERIALS AND METHODS

Patient characteristics

SCID: Thymus tissue from four children with SCID was obtained as part of an ongoing study of immunodeficiency diseases. Tissue from all children was obtained after informed consent had been given under protocols approved by the Human Subject Committees of the Universities of Wisconsin, Arizona, and Texas Medical Branch. Erythrocyte adenosine deaminase (ADA) and nucleoside phosphorylase levels (performed by R. Scott and E. Giblett, Seattle) were normal in three of four cases (patients A, B and C) and deficient in the last (patient D). This patient was unusual in that the thymic morphology was normal and that her clinically normal mother also lacked ADA [15].

DiGeorge syndrome: Thymus was obtained at autopsy from one patient (E) with DiGeorge syndrome. This child had absent T-lymphocyte activity at birth but subsequently spontaneously established immune function and died of an inoperable cardiac defect.

Normal: Thymic tissues were obtained from otherwise normal children undergoing surgery for cardiac abnormalities.

Thymus

Thymic tissues were processed as previously described [16]. Briefly, specimens were processed immediately or frozen at −80°C in TEM buffer (50 mM Tris HCl, pH 7.7, IM EDTA, 1 mM mercaptoethanol) until used. All procedures were performed at 4°C. Thymocytes were obtained by mincing the tissue into 1–2 mm^3 pieces in a mortar and subsequently suspending the pieces in RPMI 1640 without serum. Gentle agitation further released thymocytes into the medium. The cells were washed twice in fresh medium, enumerated and 10^8 cells obtained for immediate use or stored at −80°C in TEM.

Assay of TdT

1. Chemicals. [^{3}H]dGTP (15 Ci/mmol) was obtained from Schwartz-Mann Biochemicals, Orangeburg, NY; Poly(da) was obtained from P-L Biochemicals, Milwaukee, WI; cold dNTP and ATP from Sigma Biochemicals, St. Louis, MO; DEAE cellulose-52 and phosphocellulose P-11 from Whatman, Inc., Clifton, NJ; and oligodT(T-2) cellulose from Collaborative Research, Inc., Waltham, MA. All other reagents used were of the highest available reagent grade.

2. Extraction procedure. TdT was extracted from whole thymus or thymocytes by a modification of the procedure described by McCaffrey et al. [10]. One gram of normal tissue, 0.10 g of patient tissue, or 1.0×10^8 thymocytes was suspended in 5 vols. of cold TEM buffer and homogenized in a Sorval Omnimixer at half speed for 5 min. This suspension was brought to a concentration of 0.50% Triton X-100 and stirred for 1 h. The crude suspension was centrifuged at $144{,}000 \times g$ for 60 min in a Beckman type 40 rotor. The supernatant resulting from this procedure was used in the purification.

3. DEAE cellulose chromatography. The $144{,}000 \times g$ supernatant was applied directly to a DEAE cellulose column (2×5 cm) equilibrated with TEM buffer containing 20% (v/v) glycerol (TEMG). After the sample was loaded, the column was washed with TEMG buffer containing 0.30 M KCl. Fractions of 1.50 ml were collected and a 10-μl aliquot of each used as the source of enzyme for assay. The area of peak enzyme activity was pooled and dialyzed against 100 vols. of TEMG buffer for 16 h.

4. Phosphocellulose (PC) chromatography. The dialyzed enzyme preparation was applied directly to a phosphocellulose column (1.4×4 cm) equilibrated with the TEMG buffer. The column was washed with 5 column vols. of TEMG buffer and then developed with a 70 ml linear salt gradient (0.05–1.0 M KCl). A 10-μl aliquot of each 1.50 ml fraction was used as the source of enzyme.

5. OligodT cellulose chromatography. One gram of T-2 oligodT cellulose was equilibrated with 0.04 M KCl in TEMG. One to two milliliters of enzyme material in the TEMG was applied to an 8-ml column of oligodT cellulose and developed with a 100 ml 0.04 to 1.5 M KCl gradient. Two-milliliter fractions were collected and 10-μl portions used as the source of enzyme for assay [17].

For PC and oligodT chromatography, the salt concentration of every fifth fraction was determined using a conductivity meter. The peak areas of activity were pooled and stored at -80°C in small aliquots. The protein concentration of the various enzyme fractions was determined by the procedure of Lowry et al. [18].

6. TdT assays. TdT activity in the presence or absence of 75 μM ATP was measured as described previously [16]. A standard reaction mixture of 0.05 ml contained a final concentration of 80 mM Tris HCl (pH 7.5), 40 mM KCl, 0.80 mM $MnCl_2$, 0.40 mM DTT, 2.50 μCi[^{3}H]dGTP and 1.60 μg of poly(dA). dGTP was added to a final concentration of 75 μM and incubated for 1 h at 37°C. The reaction was terminated by the addition of 15 μg bovine serum albumin and 2.0 ml of 10% trichloroacetic acid (TCA) containing 0.20 M sodium pyrophosphate. The acid insoluble material was collected on a Millipore filter (0.45 μm), washed with 5.0% TCA, dried and counted in a liquid scintillation counter. All reactions were done in triplicate and the results expressed as an average of the values.

Adenosine deaminase assay

Thymic lymphocytes were minced and teased from thymus tissue in RPMI 1640. Approximately 5×10^6 cells were then broken with a Dounce homogenizer and centrifuged. The supernatant (25 μl) was then added to 30 μl of Tris-saline buffer and the reaction begun by addition of 10 μl of [8-^{14}C]adenosine concentration of 80 μM. The samples were incubated at 37°C for periods up to 1 h. The reaction was quenched by the addition of equal volumes of ethanol at 10-min intervals. Adenosine, inosine and hypoxanthine were separated on BEI cellulose with H_2O-NH_4OH (80:20 v/v). The spots were dried and counted in a liquid scintillation counter.

RESULTS

Enzyme activity

TdT activity was measured in crude extracts of thymus tissue from four patients with SCID and from one patient with DiGeorge syndrome; each was compared to the mean value of crude extracts from three age and sex-matched controls [16]. Enzyme activity was present in patients A, B, D and E and were 50, 151, 32 and 42% of control values, respectively, when the results were expressed as pmol/0.5 g wet tissue. If the results were expressed as pmol/mg protein, 84, 110, 39 and 84% of normal activity were seen, respectively. The enzyme activity in the crude extract which incorporated dGTP onto poly(dA) was inhibited by ATP (a specific inhibitor of TdT [19]) in patients A and B, but not in patient D (patient E was not studied). Patient C had no demonstrable TdT activity (Table I).

Purification procedures

In order to further analyze the enzymatic activity in patients A and B, the absence of activity in patient C material and the lack of ATP inhibition in patient D material, preparative chromatography was performed when sufficient material was available. The procedure consisted of DEAE treatment followed by phosphocellulose (PC) and/or oligodT chromatography.

After DEAE chromatography of the crude extract from patient A, enzyme activity was present in fractions 2–4 and 7–10, which were pooled (respectively A_1 and A_2). Each pool was then individually purified by PC.

TdT activity in pool A_1 was present in the 0.24 and 0.43 M KCl fraction from PC, a pattern similar to that of a normal 5-mth-old male (Fig. 1). Enzyme activity in pool A_2 was unexpectedly found in the low salt eluate (0.05 M KCl) rather than in the 0.20–0.4 M KCl range as found in thymuses of normal individuals ranging from 11 to 108 mth of age (Table II). Activity in the adjacent range (0.10–0.12 M KCl) was seen in similarly processed normal thymus extracts from a 2-day-old and 53-yr-old individual.

DEAE fractions containing polymerase activity from each of the other patients studied were individually pooled as a conservation measure prior to further purification procedures. Activity in patients B and D was also found in the low salt (0.05 M KCl) eluate after PC chromatography.

Material from patient C was not purified by PC because of limited material which

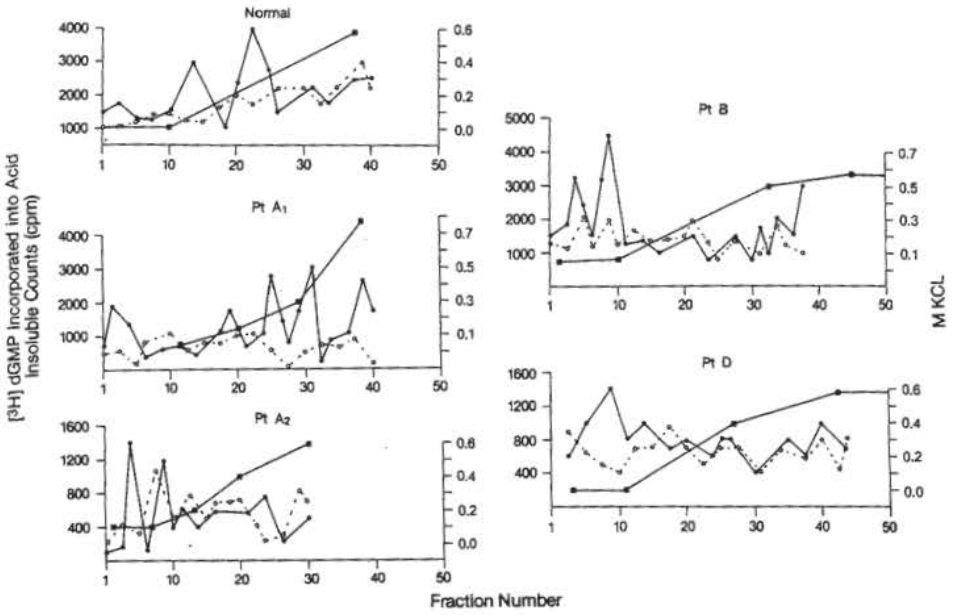

Fig. 1. Phosphocellulose chromatography patterns of thymus TdT activity with (○– – – –○) and without ATP (●————●) inhibition. Material from a normal 5-mth-old male and from pool 1 from patient A had TdT activity in the expected (0.25 M KCl (■————■) range, as well as in the 0.10 and 0.05 M KCl ranges, respectively. Enzyme activity in pool 2 of patient A, and material from patients B and D was in the low salt eluate.

was reserved for oligodT chromatography, a method known to specifically enhance the detection of TdT [17]. After this last purification step, enzyme activity inhibited by ATP was found in the 0.4–0.5 M KCl fraction in both patient C and D as well as in normal age-matched female control (Fig. 2). Activity in the purified material contrasts sharply with the undetectable (patient C) or low (patient D) levels of TdT

TABLE I

TdT in thymus of immunodeficient patients.

Diagnosis	Patient	Age (mth)	Sex	Pmol/0.5 g tissue	Pmol/ mg protein	ATP inhibition
SCID	A	3	M	445 (50) [a]	117 (84)	Yes
	B	5	M	720 (151)	23 (110)	Yes
	C	7	F	0 (0)	0 (0)	
(ADA-)	D	7	F	367 (32)	360 (39)	No
DiGeorge syndrome	E	3	F	376 (42)	96 (84)	ND

[a] Values in parentheses represent percentage of the mean of three age- and sex-matched normal controls. ND, not done.

TABLE II

Phosphocellulose purified terminal transferase from whole normal human thymuses.

Age	Sex	KCl concentration of peak enzyme activity (M)	Activity (cpm/mg)	Percentage of inhibition by ATP [a]
2 days	F	0.12	12,352	97
		0.25	12,978	96
2 days [b]		0.10	36,034	95
		0.27	117,500	98
2 mth	M	0.10	160,000	56
		0.25	56,875	24
11 mth	M	0.22	15,785	92
17 mth	M	0.28	5,694	88
38 mth	F	0.22	3,686	95
96 mth	F	0.28	66,326	91
96 mth [b]		0.24	68,901	85
108 mth	F	0.25	5,304	85
13 yr	F	0.35	11,400	71
		0.50	9,500	80
53 yr	M	0.10		85
		0.19	3,027	0
		0.25	2,596	40

[a] Inhibition was calculated by summing the area under the curve of the 1/[cpm without ATP − cpm with ATP].

[b] Activity in thymocytes separated from whole tissue.

detected in crude extracts. Likewise, ATP inhibition was readily demonstrable in the purified material but not the crude material from patients C and D.

Adenosine deaminase activity was undetectable in thymocytes from the only patient (D) studied, whereas normal thymocytes contain 200 mM per mg protein per min of enzyme activity.

DISCUSSION

Because of its unique in vitro function and relationship to precursor T-lymphoid cells and thymocytes, it has been hypothesized that TdT is associated with development of the immune system. This study examined the status of this DNA polymerase in the thymus of patients with marked immune deficiencies. We identified ATP inhibitable enzyme activity in the crude thymus extracts of two patients with SCID and in purified extracts of two additional ones with this syndrome. Similar levels of enzyme activity, although not tested with ATP, were found in crude material from a patient with DiGeorge syndrome.

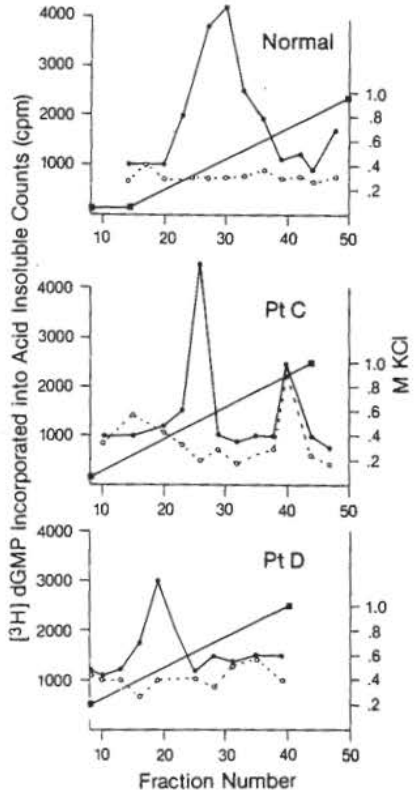

Fig. 2. OligodT chromatography patterns of TdT activity with (○----○) and without (●——●) ATP inhibition from a normal 7-mth-old female and from SCID patients C and D. Enzyme activity is in the expected 0.40–0.50 M KCl (■——■) range in each case.

If TdT is a marker of precursor T-lymphocytes in the bone marrow [20], its presence in the thymus of these patients suggests lymphocyte development past the stem cell stage and migration to the thymus. This observation strengthens previous histologic findings that thymocytes may be more prevalent during early life than at death in patients with SCID [21]. The virtually normal histology of the thymus specimen in patient D epitomizes this concept. TdT has been used as a marker of pre-T-cell development in studies in nude mice. Enzyme activity was present in the bone marrow of certain strains of nude mice [22], but not in others [23]. In the latter, the indication of TdT activity by in vitro and/or in vivo exposure to thymic hormones was strain-dependent. These findings lend credence to the hypothesis that an intrathymic or thymic hormone defect may be responsible for some types of immunodeficiency disease [24].

TdT activity was present in each patient reported, but the activity was not detected until extensive purification procedures had been undertaken. TdT activity

in various salt fractions after DEAE purification was not always inhibited by ATP. After PC chromatography, all TdT activity could be inhibited by ATP but the enzyme was present in the low salt eluate in 3 of 4 patient specimens. Following oligodT purification, specific TdT activity was normal in the 2 patients studied.

We questioned whether these results were due to non-specific proteolysis of TdT. Several observations based on study of normal thymuses argue against this possibility. The peaks of activity in different aged thymuses were consistent and if they are due to proteolytic activity, it is likely to be a specific proteolysis. Similar peaks of activity were obtained whether TdT obtained from intact thymus or separated thymocytes was purified. In addition, peaks were obtained when the proteolysis inhibitor phenylmethylsufanyl fluoride was used throughout the purification procedure (data not shown). Similar observations concerning proteolysis of this enzyme in processed animal thymuses have been reported previously [24].

Diminished TdT activity in the crude thymus extract and the presence of an altered chromatographic pattern after PC chromatographic pattern in material from patient D is of particular interest. In ADA negative SCID, the accumulation of ribonucleotides [26] and deoxyribonucleotides [27] in lymphocytes has been suggested as a mechanism of lymphocyte dysfunction [28]. The apparent specific inhibition of TdT by ATP would make this nucleotide a likely candidate causing altered enzyme expression [19]. Although deoxyATP is more toxic to lymphoid function than is ATP [29] and, therefore, is also a candidate, all dNTPs serve as substrates for TdT activity. DeoxyATP, however, is not as efficient as a substrate for TdT as is dGTP [12]. In preliminary studies, combination of dATP with dGTP in varying molar ratios reduced expected [^{3}H]dGMP incorporation suggesting an inhibitory effect. It is possible that these substances contributed to the decreased levels of TdT seen in patient D who had no ADA in her thymocytes and that purification with oligodT removed inhibitor activity. Small sample sizes, however, precluded quantitation of nucleotides in these patients. Since these nucleotides may also accumulate in patients with ADA-positive SCID [30], interference of TdT expression in patients A and C may have occurred on the same basis.

Several explanations are possible for the reduction and/or the peculiar chromatographic distribution of TdT in these patients. The most obvious is limited numbers of total or types of thymic lymphoid cells. Thymic morphology in biopsy specimens from immunodeficient patients has been described in detail [21]. When these criteria were applied in these cases, patients A and C fell into the totally dysplastic group, while patients B and D were in the partially dysplastic and normal groups, respectively. The thymus of patient E had a morphologic pattern typical of DiGeorge syndrome [2]. At first glance, the number of lymphoid cells in the thymuses of patients A through C appears to correlate directly with crude TdT activity. This correlation does not hold in patient D. Furthermore, because TdT activity after phosphocellular chromatography was found in the eluate fraction irrespective of thymic morphology, it is doubtful that differences in lymphocyte numbers per se account for these findings. Partial classification of thymic cell types was possible in patient D. Ninety-eight percent (normal $>90\%$) and $>2\%$ (normal range unknown) of thymocytes formed E or EAC rosettes, respectively (unpubl. results). These

normal data, although limited, do not correspond with TdT activity or expression, therefore making this explanation unsatisfactory as well.

Additional or altered forms of TdT and/or migration of selected lymphoid precursors contained altered or immature forms of TdT to the thymus are also possible explanations. Bollum and Brown identified a high molecular weight form of TdT and have predicted other variants [31]. We have previously shown TdT activity in less dense, immature thymocytes as well as in the more mature, dense small thymocytes which were E-rosette positive in normal thymuses [32]. Age- and sex-dependent levels of TdT in normal thymuses [16] and the age-related chromatographic patterns in neonates and older individuals observed here implicate changes in lymphoid populations within the thymus throughout life.

An additional hypothesis in keeping with the above is that expression of TdT activity may be controlled by nonlymphoid elements [33] within the thymus. The increased TdT activity in PG-purified thymocyte extracts (vs. whole tissue extracts) from a 2-day-old normal infant, but not from a normal 96-mth-old child (Table II) supports this concept. Preliminary data from experiments where extracts from whole thymus were mixed with extracts from thymocytes showed a reduction in expected TdT activity. Control of TdT activity within the thymus by any of the proposed mechanisms is conceptually appealing since in man TdT activity is normally limited to precursor lymphocytes in the bone marrow and in cortical thymocytes. Either TdT bearing cells do not leave the thymus or during maturation or migration to peripheral blood and other organized lymphoid organs they lose ability to express enzyme activity. The results reported in this initial study support the need for further evaluation of TdT activity in immunodeficient patients and examination of factors which may influence its expression.

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