# TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE IS FOUND IN PROTHYMOCYTES*

By ALLEN E. SILVERSTONE,‡ HARVEY CANTOR,§ GIDEON GOLDSTEIN, AND DAVID BALTIMORE‖

*(From the Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139; the Department of Medicine of Harvard Medical School and the Sidney Farber Cancer Center, Boston, Massachusetts 02115; and the Memorial Sloan-Kettering Cancer Center, New York 10021)*

Terminal deoxynucleotidyl transferase is an enzyme which has the unique property of polymerizing polydeoxynucleotides onto a primer in the absence of a template (1, 2). This enzyme is found both in the thymus and the bone marrow of birds, rodents, and humans (3-7). Whether the marrow cells that contain terminal transferase are related to thymocytes, or are on a separate pathway of differentiation, is not yet known (7, 8).

To determine the lineage of the murine bone marrow cells that have terminal transferase, we have investigated whether these cells have the antigen Thy-1 which is characteristic of T cells and thymocytes, or whether Thy-1 can be induced on the cells by treatment with thymopoietin (9). Thymopoietin is known to induce a set of characteristic T-cell markers including the Thy-1 alloantigen on the surface of a subpopulation of bone marrow cells committed to T-cell differentiation (prothymocytes) (10). Destruction of Thy-1-positive cells after exposure to thymopoietin allows elimination of a substantial fraction of those bone marrow cells that can repopulate an irradiated thymus (11). We find that such an elimination after induction with the thymic polypeptide removes a substantial amount of terminal transferase from the bone marrow cell population, suggesting that at least one-half of the marrow cells bearing this enzyme are related to those found in the thymus.

## Materials and Methods

*Mice.* C57BL6/J and A/J mice from The Jackson Laboratory, Bar Harbor, Maine, were used.

*Cell Preparation.* Thymocytes and marrow lymphocytes extracted from femoral and tibial plugs were suspended in phosphate-buffered saline (PBS)-5% fetal calf serum (FCS) (Microbiological Associates, Bethesda, Md.) as described (7).

*Induction of Cytotoxic Antibodies.* Bovine thymopoietin was prepared as described (9). It was dissolved in PBS-2% FCS at a final concentration of 500 ng/ml. In a typical experiment cells were incubated at $4 \times 10^7$/ml for 2.5 h at 37°C, then washed twice with PBS-2% FCS.

*Complement-Mediated Cytotoxicity.*   Anti-Thy-1.2 was either a gift of Dr. E. A. Boyse (Memorial Sloan-Kettering Cancer Center, New York) or was prepared as described (12). The method of treatment of lymphoid cells with anti-Thy-1.2 serum and complement (C) has been described previously (12). In a representative experiment, $10^8$ cells/ml were incubated with Thy-1.2 antiserum at a dilution of 1:50 at room temperature for 30 min, washed once, and incubated for a further $1/2$ h with rabbit C (1:10 dilution) at 37°C. The cells were then pelleted and washed several times. In cases where viability was less than 85%, Ficol-Hypaque centrifugation was used to remove dead cells (13), before the cells were pelleted and frozen at −70°C.

*Extraction of Terminal Transferase.*   The procedure has been previously described (7), except that in order to inhibit protease activity the inhibitor phenylmethylsulfonylfluoride (PMSF) (Sigma Chemical Co., St. Louis, Mo.) was introduced at the time of the first thawing of the sample at a concentration of 4 $\mu$mol/$10^8$ cells in 4.3 mmol of ethanol (per 5-ml vol).

*Phosphocellulose Chromatography.*   The procedure was modified (5, 7) to examine the smaller amounts of activity present in the bone marrow. A 4 cm column, rather than 12 cm was used, the gradient was only 25 ml in total vol, and fractions of 0.25–0.32 ml size were collected. Terminal transferase assays were done as previously described (5, 7).

## Results

To determine the effect of anti-Thy-1.2 and C upon terminal transferase activity in thymocytes, the amount of terminal transferase in the cells remaining after treatment was measured and expressed as specific activity of enzyme per $10^9$ remaining viable cells. Although anti-Thy-1.2 serum and C killed most thymocytes (94% were specifically killed by antiserum in exp. 1, Table I), the remaining cells had the same activity per cell of terminal transferase as cells treated with normal mouse serum (Table I, lines 1 and 2). This level of enzyme activity is similar to that in untreated thymocytes (reference 7 and unpublished data). The few anti-Thy-1.2-resistant cells are therefore not different from the average normal thymocyte population.

The effect of anti-Thy-1.2 treatment on the terminal transferase content of the marrow lymphocytes was studied using the same protocol used for the thymocytes. In the marrow most cells lack detectable terminal transferase (P. C. Kung, unpublished results) and therefore if the 2–5% Thy-1-positive cells in bone marrow (10, 11) were responsible for all of the marrow terminal transferase, anti-Thy-1.2 serum should greatly reduce the specific activity of the enzyme in marrow cells. On the contrary, terminal transferase activity was unchanged by treatment of marrow cells with anti-Thy-1.2 and C (Table I, lines 1, 2, 4, 7, and 8). The majority of the enzyme-positive cells of the marrow therefore lack Thy-1 determinants on their surface.

To determine if Thy-1 determinants could be induced onto the surface of the terminal transferase-containing cells, marrow cells were treated with thymopoietin for 2–3 h. When the thymopoietin-treated cells were exposed to anti-Thy-1.2 antibody, the terminal transferase specific activity was reduced by over 50% (Table I, lines 3, 6, and 10). Because treatment with thymopoietin alone did not alter the terminal transferase content of the marrow cells (Table I, lines 5 and 9) it appears that thymopoietin induced Thy-1.2 expression on the surface of a significant portion of the terminal transferase-containing cells, thus making them susceptible to the antiserum. The number of cells specifically killed in the marrow by anti-Thy-1.2 treatment was impossible to determine with accuracy because there was a high background killing by C alone. In some cases (exps. 1

Table I

*Effect of Antibody Treatment on Cell Number and Terminal Transferase Levels in C57BL/6J and A/J Mice*

| | Inductive treatment | Postinduction antiserum + C | Terminal transferase remaining | |
|---|---|---|---|---|
| | | | Thymocytes | Bone marrow lymphocytes |
| | | | $U/10^9$ cells | |
| Exp. 1, C57BL/6J | None | Normal mouse serum | 2.69 | 0.208 |
| | None | Anti-Thy-1.2 | 2.50 | 0.188 |
| | Thymopoietin | Anti-Thy-1.2 | – | 0.061 |
| Exp. 2, A/J | None | Anti-Thy-1.2 | – | 0.216 |
| | Thymopoietin | Normal mouse serum | – | 0.204 |
| | Thymopoietin | Anti-Thy-1.2 | – | 0.107 |
| Exp. 3, A/J | None | Normal mouse serum | – | 0.206 |
| | None | Anti-Thy-1.2 | – | 0.243 |
| | Thymopoietin | Normal mouse serum | – | 0.198 |
| | Thymopoietin | Anti-Thy-1.2 | – | 0.102 |

and 2, Table I) 5–10% specific killing over the C background could be detected, but in experiment three (Table I) no specific killing over background was seen.

We have previously noted that thymocytes contain two forms of terminal transferase, one of which elutes from phosphocellulose at lower salt concentration than the other (7, 8). We have also found that well-defined two-peak profiles of elution from phosphocellulose are found most reproducibly if a protease inhibitor, PMSF, is added to the extraction medium (unpublished observations). Even using the protease inhibitor, bone marrow terminal transferase did not give a two-peak profile of elution (Fig. 1). The majority of the enzyme, however, elutes in the region of the column where the low salt, "peak I" form of terminal transferase elutes (compare figures in reference 7). Treatment of cells with thymopoietin followed by anti-Thy-1.2 serum produced a generalized decrease of terminal transferase throughout the phosphocellulose elution profile (Fig. 1) while neither anti-Thy-1.2 treatment alone or thymopoietin alone had any reproducible effect. In the experiment of Fig. 1, a 75% reduction of terminal transferase was produced by thymopoietin plus anti-Thy-1.2 treatment; this is the largest effect we have seen.

Table II presents the averaging of the results of five experiments. It is clear that although there is no significant loss of marrow terminal transferase after anti-Thy-1.2 treatment, or C treatment, of mouse bone marrow cells, the elimination of Thy-1.2 cells after induction with thymopoietin results in a significant decrease of terminal transferase. This loss represents an average of 58% with a range of loss of enzyme from 50 to 75%.

## Discussion

In order to investigate the nature of the bone marrow cells containing terminal transferase, we have taken advantage of the ability to induce a thymus-specific antigen, Thy-1, on bone marrow cells using the thymic polypeptide, thymopoietin. After this treatment, cells now expressing the antigen can be killed by cytotoxic antibody treatment. As our results show, this leads to a destruction of marrow cells carrying terminal transferase. Therefore, a signifi-
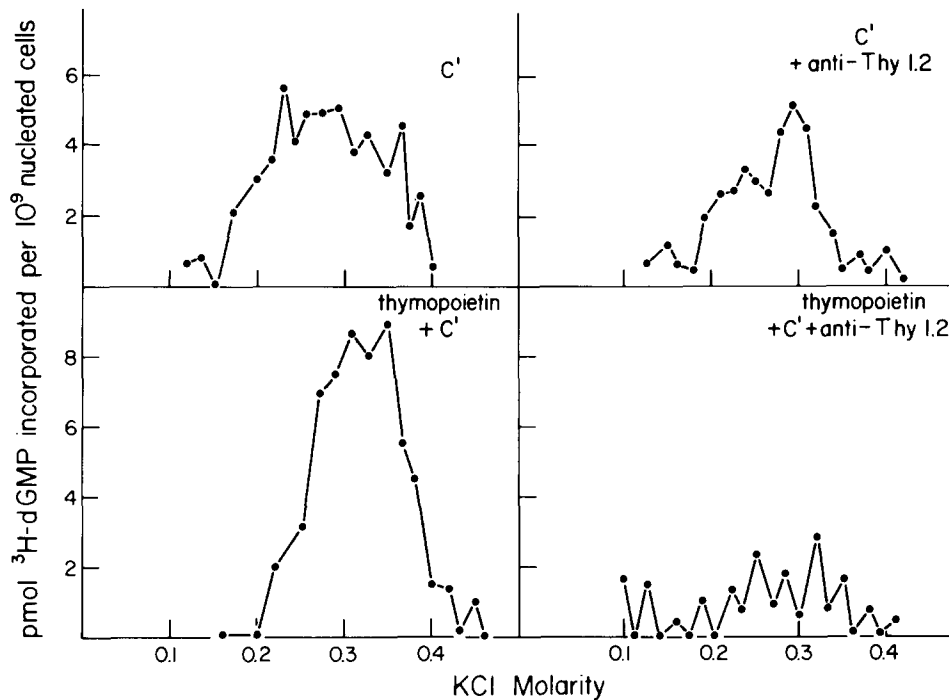
FIG. 1.   Elimination of terminal transferase-bearing cells in bone marrow by induction and cytotoxic antibodies. C', C-treated samples. Anti-Thy-1.2 treated with antiserum. Treatment is described in the Materials and Methods. See text for protocol.

TABLE II

*Average Results of Induction and Cytotoxic Elimination of Terminal Transferase Levels in Marrow Cells*

| Inductive treatment | Postinductive treatment | Terminal transferase remaining* |
|---|---|---|
| | | $U/10^9$ cells |
| None | None | 0.208 |
| None | Normal mouse serum | 0.216 |
| None | Anti-Thy-1.2 + C | 0.191 |
| Thymopoietin | Normal mouse serum + complement | 0.219 |
| Thymopoietin | Anti-Thy-1.2 + C | 0.090 |

*Each value represents average of three or four determinations, with standard deviation less than 20%.

cant portion of the cells in the mouse marrow which bear terminal transferase must already be committed to the T-cell pathway. Since thymopoietin appears to selectively induce a population already committed to development along the T-cell pathway (14), and does not affect lymphocytes destined for the B-cell pathway, the population of cells we have eliminated probably represents a portion of the prothymocyte population of the marrow.

In earlier papers (7, 8, 15) it has been suggested that the marrow cells bearing terminal transferase are related developmentally to the thymus cells which bear high levels of this enzyme. Here, we present data supporting this supposition. Whether all the marrow cells bearing terminal transferase are destined to enter

the T-cell pathway is uncertain. In our experiments we may not have eliminated all the measurable terminal transferase because of the incompleteness of our induction or incompleteness of killing by cytotoxic antibody, or both. We are unable to settle this question given the current limits in our technique. However, we are investigating the possibility that terminal transferase may also be present in pre-B cells using an experimental approach similar to that described here.

We can say from our results that terminal transferase is a valuable marker for studying T-cell development. It is now the earliest known property of cells that can undergo thymus-dependent development, and it clearly disappears before the final maturation of the T cell to a circulating lymphocyte (15, 16). Studying the appearance and disappearance of this enzyme in selected cell populations may help explain the development of function by T cells.

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