Intracellular distribution of terminal deoxynucleotidyl transferase in rat bone marrow and thymus

(nuclear and cytoplasmic terminal transferase/immunofluorescence/Thy-1.1 antigen/thymocyte progenitors/
DNA nucleotidylexotransferase)

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ABSTRACT A subset of bone marrow cells that contains terminal deoxynucleotidyl transferase (DNA nucleotidylexotransferase; nucleosidetriphosphate:DNA deoxynucleotidylexotransferase, EC 2.7.7.31) can be identified in adult rats by immunofluorescence using affinity-column-purified antibody to homogeneous calf transferase. The transferase-positive cells comprise approximately 1.8% of bone marrow cells. Correcting the specific activity of terminal transferase in total bone marrow cells (0.21 units per 10^6 cells) for the percentage of transferase-positive bone marrow cells (1.8%) gives 1.17 units per 10^6 cells, a value approximately half that found for transferase-positive thymocytes.

Fluorescence appears to be restricted to the nucleus of transferase-positive bone marrow cells, in contrast to the predominantly cytoplasmic fluorescence of small thymocytes from adult rats. Some large thymocytes contain intranuclear transferase fluorescence patterns similar to those seen in bone marrow. These thymocytes are especially numerous in neonatal rat thymus, where they are localized in the subcapsular region of the cortex. Thymocytes with combined patterns of nuclear and cytoplasmic transferase are also present. In addition, Thy-1.1 antigen, which is present on thymic and prethymic cells but not on the majority of post-thymic cells in the rat, is also present on transferase-positive bone marrow cells.

The results suggest that the transferase-positive subset of bone marrow cells may contain the immediate progenitors of cortical thymocytes in the rat. The nuclear location of fluorescence may indicate the site of physiological activity of terminal transferase in thymocytes and their precursors.

Terminal deoxynucleotidyl transferase (DNA nucleotidylexotransferase; nucleosidetriphosphate:DNA deoxynucleotidylexotransferase, EC 2.7.7.31) is an enzyme that has the unique capacity to add mononucleotides to any 3'-OH-terminated segment of DNA in the absence of a template (1). It has been found in high concentrations in thymus and in low concentrations in bone marrow of all species tested, but not at all in the avian bursa of Fabricius or in peripheral lymphoid tissues (2-6). Consequently, terminal transferase has been postulated to participate in the early stages of T lymphocyte differentiation, possibly as a somatic mutagen in the generation of immunological diversity (7, 8). Terminal transferase has been identified in neoplastic leukocytes from a variety of leukemic disorders of man and mouse (3, 9-13).

In our original study of transferase immunofluorescence, we demonstrated fluorescence predominantly within the cytoplasm of cortical thymocytes from rat, mouse, and calf (14). No fluorescence was detected in medullary thymocytes or in peripheral T or B cells. Using similar techniques, we now report the presence of terminal transferase within the nucleus of rat bone marrow cells and immature cortical thymocytes.

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MATERIAL AND METHODS

Lymphoid Tissues. Thymus and/or bone marrow were obtained from neonatal and 8- to 10-week-old male and female (Lewis × DA) F1 strain hybrid rats. Frozen sections and cell suspensions were prepared as described previously (15).

Antisera. The method of preparation and properties of rabbit antiserum to homogeneous calf terminal transferase have been described elsewhere (14, 16). The antiserum obtained cross-reacts strongly with rat terminal transferase. In the present experiments, an IgG fraction of rabbit anti-transferase (lot no. 53) was rendered highly specific by elution from a terminal transferase immunoadsorbent column (see below). The column-purified anti-transferase was used for immunofluorescence at a concentration of 200 µg or 400 µg of protein per ml of Dulbecco's phosphate-buffered saline.

The immunoadsorbent column was prepared by aldehyde coupling of homogeneous calf terminal transferase to controlled pore glass (Corning CPG 550, glycolphase obtained from Pierce Chemical Co., Rockford, IL). About 5 mg of transferase was mixed with 150 mg of periodate-activated CPG 550 glycolphase and greater than 90% coupling was achieved after NaBH4 reduction. Terminal transferase coupled to CPG 550 was enzymatically active. Crude rabbit anti-transferase IgG was applied to the transferase-CPG 550 column in phosphate-buffered saline. After extensive washing with saline the pure anti-transferase IgG was eluted with 0.5 M NaCl containing 50 mM glycine at pH 3.5. The eluted fractions were neutralized with 1 M Tris-HCl, pH 8.8, and tested for enzyme neutralization and precipitin formation. Fractions containing more than 100 µg of protein per ml were pooled, dialyzed against 10 mM potassium phosphate, pH 7.2, and lyophilized.

Antiserum to Thy-1.1 antigen, prepared in congenic strains of mice (AKR/Cum against AKR/J) (17), was a gift of Roger Morris. Thy-1.1 antigen has been shown to be present on rat brain, thymus, and bone marrow cells (18).

Rhodamine-conjugated goat IgG against mouse IgG and fluorescein-conjugated goat IgG against rabbit IgG were obtained from Cappel Laboratories, Downingtown, PA. The rhodamine-conjugated goat anti-mouse IgG was adsorbed with rat serum affixed to a column of CNBr-activated Sepharose 4B (courtesy of Roger Morris).

Immunofluorescence. Cell suspensions were distributed on glass slides in a cytocentrifuge (Cytospin, Shandon Instruments, Sewickley, PA) using concentrations of 5 × 10^6 cells per ml of fetal calf serum (50% vol/vol in Tyrode's solution). Cytospin preparations and frozen sections of thymus were fixed (5 min,
Fig. 1. Adult rat bone marrow cells. Cells were fixed in methanol, incubated with rabbit anti-transferase, and developed with fluorescein-conjugated goat anti-rabbit IgG. (X775.) (A) Two small lymphocyte-like cells show coarsely granular nuclear fluorescence. The cytoplasm is difficult to discern in the photograph, but appeared not to fluoresce microscopically. Several nonfluorescing bone marrow cells are visible in the field as pale "ghosts." (B and C) Medium-size cells show intense, coarsely clumped nuclear fluorescence. There is no detectable cytoplasmic fluorescence. Two nucleoli in C are visible as non-staining "holes" in a brightly fluorescing nucleus. (D) Large cell with diffuse, finely stippled pattern of nuclear fluorescence.

4°C in 95% ethanol or absolute methanol and processed for indirect immunofluorescence as previously described (14).

Immunofluorescence for terminal transferase and Thy-1.1 antigens was simultaneously tested on alcohol-fixed bone marrow smears by sequential incubation at room temperature with the following antisera: rabbit anti-transferase (20 min); fluorescein-conjugated goat anti-rabbit IgG (15 min); mouse anti-Thy 1.1 (15 min) and rhodamine-conjugated goat antimouse IgG (10 min). Each incubation period was followed by two washes in Dulbecco's phosphate-buffered saline (5 min each). The processed cell smears were mounted with buffered glycerol (pH 7.2) and individual cells were observed for fluorescence using narrow-band filters selective for rhodamine and fluorescein (Zeiss Universal microscope with epi-illumination system). Control slides, containing cells treated with antitransferase or anti-Thy-1.1 only, were developed with the appropriate or inappropriate fluorescent antiserum. Normal mouse serum and normal rabbit serum controls were also included.

Enzyme Assay. Sonicated thymus cell suspensions were assayed for terminal transferase activity as described previously (19). Bone marrow cell suspensions were assayed by the same method using 0.1 mM [3H]dGTP at 300-500 cpm/pmol. A unit of enzyme catalyzes transfer of 1 nmol of deoxynucleotide to DNA per 60 min under the standard reaction conditions.

RESULTS
Identification of Transferase-Positive Bone Marrow Cells by Immunofluorescence. Bone marrow cells from normal adult rats were incubated with affinity-column-purified rabbit anti-transferase and were developed for indirect immunofluorescence. The anti-transferase did not react with the surface of viable bone marrow cells. However, it did react strongly with a small subset (1.80 ± 0.14%) of bone marrow cells in alcohol-fixed smears. The fluorescence appeared to be restricted to the nucleus of the transferase-positive cells (Fig. 1). Small and medium-sized, lymphocyte-like bone marrow cells showed a coarsely clumped pattern of intranuclear fluorescence that resembled the aggregated pattern of heterochromatin seen in Wright's stained preparations. Some large, blast-like bone marrow cells exhibited a more diffuse, finely granular pattern of intranuclear fluorescence that resembled the dispersed chromatin pattern seen in these cells.

Thy-1.1 Antigen on Transferase-Positive Bone Marrow Cells. Adult rat bone marrow cells were tested for the simultaneous presence of Thy-1.1 and transferase antigens by dual immunofluorescence. Anti-Thy-1.1 staining (rhodamine-positive) was restricted to the cell surface; anti-transferase staining (fluorescein-positive) was restricted to the cell nucleus. Approximately 30% of nucleated bone marrow cells were found to be Thy-1.1 positive, as reported elsewhere (18). Of these, approximately 10% were transferase-positive (i.e., showed dual fluorescence). Conversely, essentially all transferase-positive bone marrow cells were Thy-1.1 positive.

Intranuclear Distribution of Terminal Transferase in Thymocytes. As we and others have reported (2, 14), terminal transferase appears to occupy a predominantly cytoplasmic position in cortical thymocytes from adult rats. The use of a Cytospin centrifuge to prepare cell smears and the use of affinity-column-purified anti-transferase for immunofluorescence permitted more precise resolution of the intracellular distribution of this enzyme in the present experiments. In the flattened preparation from the cytocentrifuge, many small thymocytes had detectable nuclear fluorescence as well as cytoplasmic fluorescence (Fig. 2A). Some large, immature thymocytes had the nuclear pattern of fluorescence reminiscent of that seen in transferase-positive bone marrow cells (Fig. 2B and C). These large thymocytes lacked detectable cytoplasmic fluorescence.

In order to examine this class of large thymocytes more carefully, cytocentrifuge preparations containing numerous immature thymocytes were prepared from thymus of neonatal rats. Many of the large and medium-sized thymocytes in these preparations were found to have abundant nuclear fluorescence and to lack detectable cytoplasmic fluorescence.

Additional patterns of fluorescence were seen in small thymocytes from newborn rats. Some small cells showed the pre-
dominantly cytoplasmic pattern characteristic of thymocytes from adult rats. Others displayed a predominantly nuclear pattern, with brightly fluorescing patches of transferase in a ring-like arrangement at the periphery of the nucleus (Fig. 3). Still other cells displayed combined nuclear and cytoplasmic patterns of fluorescence.

The anatomical locations of thymocytes that displayed predominantly nuclear or predominantly cytoplasmic patterns of terminal transferase were studied on alcohol-fixed frozen sections of thymus from newborn rats. As in adult rats (14, 19), all transferase-positive cells were restricted to the thymus cortex. Large thymocytes having the nuclear pattern of fluorescence were located in the subcapsular region of the cortex, where they formed a layer several cells deep (Fig. 4). Small thymocytes that had a predominantly cytoplasmic pattern of fluorescence were present in the inner part of the cortex. Intermediate patterns of fluorescence that were evident in cell smears could not be resolved in frozen sections.

**Enzymatic Analysis of Terminal Transferase in Bone Marrow and Thymus.** Aliquots of adult rat bone marrow and thymus cells from the preceding experiments were assayed for transferase activity. Mean terminal transferase levels ±SD were 0.21 ± 0.02 units per 10⁶ bone marrow cells and 13.77 ± 1.11 units per 10⁶ thymocytes.

**DISCUSSION**

The most intriguing observation in the present study is the difference in intracellular localization of terminal transferase in bone marrow cells and in small thymocytes. Previous biochemical and immunofluorescence studies had indicated that transferase was located predominantly in the cytoplasm of small thymocytes from adult animals (2, 14). This presented a vexing problem in attempting to associate the physiological functions of terminal transferase with DNA modification. The detection of transferase within the nucleus, but not the cytoplasm, of bone marrow cells and immature thymocytes now provides a more rational basis for such speculation. We therefore propose that terminal transferase functions in the nucleus of cortical thymocytes and thymocyte progenitors. Cytoplasmic transferase, although enzymatically active in vitro, may not be functionally active in vivo.

We have also noted that the intracellular localization of terminal transferase varies with the phase of the cell cycle (unpublished observations). During mitosis there appears to be a shift of transferase from nucleus to cytoplasm, whereas a variety of nuclear patterns of transferase immunofluorescence are seen at other points in the cycle. Dual patterns of nuclear and cytoplasmic fluorescence are commonly found among cortical thymocytes from neonatal rats. Cytoplasmic fluorescence predominates in thymocytes from adult rats. Inasmuch as immature thymocytes undergo six to eight divisions in the course of differentiation (20), it is possible that terminal transferase acts intermittently to affect individual generations of daughter cells during specific phases of the cell cycle. Such a mechanism would be compatible with the postulated role of terminal transferase as a somatic mutagen (7, 8). In addition, some of the patterns of intracellular localization of terminal transferase may signify discrete stages in the differentiation pathways of thymocytes.

The above difference in intracellular distribution of terminal transferase in bone marrow cells and thymocytes may be associated with a quantitative difference in enzymatic activity in these two cell populations. This was determined by dividing the specific activities of terminal transferase in bone marrow (0.21 units per 10⁶ cells) and in thymus (13.8 units per 10⁶ cells) by the corresponding percentages of transferase-positive cells in these tissues. Approximately 1.8% of adult rat bone marrow cells and approximately 64% of adult rat thymocytes (14) have been found to be transferase-positive. Thus, the corrected specific activities of terminal transferase per 10⁶ transferase-positive cells in bone marrow and thymus are 11.7 units and 21.6 units, respectively. This difference in specific activity was not unexpected in view of present and previous (14, 19) evidence of heterogeneity of terminal transferase content among bone marrow cells and cortical thymocytes. The higher specific activity in the thymocytes may be due to the presence of terminal transferase in both nucleus and cytoplasm.

It seems probable, in view of the restricted cellular distribution of terminal transferase, that some transferase-positive bone marrow cells are progenitors of thymocytes. Indirect evidence in support of this hypothesis recently has been presented for the mouse (21). The present findings in the rat also favor this interpretation. Thus, we have determined by dual immunofluorescence that transferase-positive cells in rat bone marrow bear the Thy-1.1 antigen. Unlike the situation in the mouse, Thy-1.1 antigen is normally expressed on lymphohemopoietic precursor cells in rat bone marrow (I. Goldschneider and R. Morris, unpublished observations), but not on the vast majority of peripheral T cells (22). The fact that transferase-positive cells constitute only about 10% of Thy-1.1 positive cells in rat bone marrow suggests that transferase-positive cells may represent a subset of precursor cells. That many of these thymocyte progenitors is supported by the presence in thymus cortex of morphologically similar cells with intranuclear transferase. The
latter cells had the appearance of large, undifferentiated lymphoblasts. Their presence in the subcapsular region of thymus cortex suggests that they bear a parent–progeny relationship with more differentiated transferase-positive thymocytes in the deeper part of the cortex (23).

We have also identified transferase-positive cells in rat spleen during the first 6 weeks of life, but not thereafter (unpublished observations). Like their counterparts in bone marrow and in the subcapsular region of thymus cortex, transferase-positive spleen cells have a nuclear pattern of fluorescence. Moreover, the age-related decrease of transferase-positive spleen cells in the rat is reminiscent of the age-related decrease of spleen hematopoietic tissues, and that transferase-positive cells in the mus (24).

The results, therefore, support the hypothetical model of thymocyte differentiation that we have presented previously (19). In that model, four subsets of cortical thymocytes were distinguished on the basis of differences in buoyant density, spontaneous incorporation of [3H]thymidine, and terminal transferase activity. It was predicted that the least dense subset of cortical thymocytes would prove to be immature, transferase-positive cells that were recently derived from transferase-positive progenitors in hematopoietic tissues.

While this is the most likely explanation of our findings, it must be cautioned that the possibility has not been excluded that some transferase-positive bone marrow cells are post-thymic rather than prethymic cells. Order and Waksman (25) have shown that some immature cells in regenerating thymus have the ability to proliferate in bone marrow after intravenous injection. It is not known if such cells are transferase-positive and if they are able to leave the thymus under physiological conditions. We have found that transferase-positive thymocytes arise earlier in ontogeny than do transferase-positive cells in hematopoietic tissues, and that transferase-positive thymocytes in irradiated adult rats can develop from transferase-negative progenitors in adoptively transferred fetal liver (unpublished observations). Although these observations indicate that under some circumstances the expression of terminal transferase may occur first in the thymus, they do not argue against the prothymocyte nature of transferase-positive bone marrow cells. Indeed, our recent findings of normal concentrations of terminal transferase and of normal numbers of transferase-positive cells in bone marrow of congenitally athymic (nu/nu) mice and of neonatally thymectomized rats (26 and unpublished observations) indicate that the contribution of thymus-derived cells to the pool of transferase-positive bone marrow cells is negligible.

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