Demonstration of terminal deoxynucleotidyl transferase in thymocytes by immunofluorescence

(cortical thymocytes/medullary thymocytes/lymph node lymphocytes/antiserum to terminal transferase)

IRVING GOLDSCHNEIDER*, KATHLEEN E. GREGOIRE*, RANDALL W. BARTON*, and F. J. BOLLUM†

* Department of Pathology, University of Connecticut Health Center, Farmington, Conn. 06032; and † Department of Biochemistry, University of Kentucky Medical Center, Lexington, Ky. 40506

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ABSTRACT The cellular and subcellular distribution of terminal deoxynucleotidyl transferase (DNA nucleotidyldeoxynucleotidyl transferase; nucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.31) in thymocytes and peripheral lymphocytes from rat, mouse, and calf was studied by immunofluorescence using rabbit antiserum to homogeneous transferase from calf. Terminal transferase was readily detected in approximately 75% of cortical thymocytes, but not in medullary thymocytes or lymph node lymphocytes. The enzyme appeared to be present predominantly in the cytoplasm of positive thymocytes in ethanol-fixed cell smears and frozen sections. The reactivity of anti-terminal-transferase for thymocytes could be neutralized with purified calf enzyme. Results of experiments in which thymocytes were separated on 7-step discontinuous Ficoll density gradients suggested that cortical thymocytes are heterogeneous with respect to terminal deoxynucleotidyl transferase content.

Terminal deoxynucleotidyl transferase (DNA nucleotidyldeoxynucleotidyltransferase; nucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.31) is an enzyme that catalyzes synthesis of deoxynucleotide sequences without template direction (1) and that appears to be normally restricted to thymus and bone marrow (2–6). This transferase has also been found in neoplastic cells from human patients with acute lymphocytic leukemia and in several thymus-derived (T) cell leukemias and lymphoma cell lines from man and mouse (7–10, 11). Transferase has not been identified in normal or neoplastic cells expressing bone-marrow-derived (B) cell characteristics.

Previous functional and antigenic analyses of thymocyte suspensions separated on discontinuous density gradients suggested that cortical thymocytes were transferase-positive (4, 11, 12). Medullary thymocytes were judged to be transferase-negative (12). We have now directly confirmed these impressions by using rabbit antiserum to homogeneous calf thymus transferase (13) to demonstrate transferase within the cytoplasm of cortical thymocytes from rat, mouse, and calf.

METHODS

Lymphoid Tissues. Thymus, cervical lymph nodes, and blood were obtained from 8- to 12-week-old male and female (Lewis × DA)F; strain hybrid rats and 8-week-old C3H and AKR strain mice. Bovine thymus and bronchial lymph nodes were obtained from a freshly slaughtered, 6-month-old female Holstein calf. Frozen sections and cell suspensions were prepared as described previously (14). In some experiments thymocytes were separated on a 7-step discontinuous Ficoll density gradient (8–20%, wt/vol) (12) before immunofluorescent staining.

Antiseras. Two lots of rabbit antiserum (no. 48 and no. 53) prepared against glutaraldehyde cross-linked homogeneous calf transferase were used (13). F(ab′)2 fragments of these antisera were prepared from purified IgG fractions by controlled pepsin digestion (cf. ref. 15). Crude IgG isolated by (NH4)2SO4 fractionation of serum was purified on Sephadex G-200. The IgG fraction from Sephadex G-200 was concentrated to 20 mg/ml, adjusted to pH 4.5 (sodium acetate buffer), and digested with 2 mg of twice-crystallized hog pepsin (Worthington) per 100 mg of IgG protein. Progress of the digestion was followed by analyzing aliquots of the digestion mixture on sodium dodecyl sulfate/polyacrylamide slab gels and observing the loss of staining intensity in the heavy chain region. Aliquots of reaction mixture were also analyzed by microimmunoelcrophoresis in 1% agarose (50 mM sodium diethylbarbiturate buffer, pH 8.4) and testing with goat antiserum to rabbit IgG and goat antiserum to rabbit Fc (gift of T. R. Rozman). In this analysis the loss of Fc reactivity and change in mobility of the IgG reaction signifies conversion to F(ab′)2. Generally speaking, 16 hr of digestion followed by addition of another 2 mg of pepsin and a further 6 hr digestion was required to obtain complete conversion to F(ab′)2.

The pepsin digests were fractionated on Sephadex G-200 and the F(ab′)2 obtained was dialyzed against 10 mM potassium phosphate at pH 7.2. The dialyzed F(ab′)2 was then passed through a 1 × 10 cm column of Sephadex A-25 and washed through with 10 mM phosphate buffer. The nonadsorbed fractions containing F(ab′)2 and antiterminal-transferase activity were lyophilized or used directly.

Aliquots of the antiterminal-transferase were diluted 1:5 in Dulbecco’s phosphate-buffered saline (containing 20 mM NaNO3) and absorbed twice (4°, 20 min) with equal volumes of packed rat erythrocytes. Additional absorptions were performed with viable rat lymph node cells or thymocytes or with purified calf transferase as indicated under Results. Absorption with transferase (gift of L. M. S. Chang) was performed by adding 1000 units (1 unit catalyzes polymerization of 1 nmol of nucleotide per hr under standard assay conditions) of homogeneous transferase (10 µl) to 100 µl of clarified antiserum and then diluting to 500 µl with phosphate-buffered saline. The mixture was allowed to stand at 4° for 4 hr and was again clarified at 100,000 × g for 60 min. Dilutions of the supernatant fraction were used directly as “transferase-absorbed” antiserum.

Rabbit antiserum to the rat bone marrow lymphocyte antigen (anti-BMLA) was used to identify cortical thymocytes (16). Lewis strain rat antiserum to DA strain rat histocompatibility antigens (Le anti-DA) was used to identify medullary thymocytes (12).

Fluorescein-conjugated rabbit IgG against rat IgG (FITC-anti-rat IgG), fluorescein-conjugated goat IgG against rabbit IgG (FITC-anti-rabbit IgG), and goat IgG against rabbit IgG
were obtained from Cappel Laboratories, Downingtown, Pa.

Immunofluorescence. Suspensions of living cells and frozen sections of fresh tissues were processed for indirect immunofluorescence as described previously (14). Appropriately absorbed preimmunization rabbit serum and IgG and F(ab')2 fractions of normal rabbit serum were used as controls for the corresponding antiterminal-transferase preparations. Cell smears were made on alcohol-cleaned glass slides, using a 3% suspension (vol/vol) of packed cells in Tyrode's solution containing 50% (vol/vol) fetal calf serum. The frozen sections and cell smears were fixed in 95% ethanol (4°C, 5 min) and were washed twice in phosphate-buffered saline prior to reacting with antisera. Absolute methanol was also a suitable fixative. Acetone, 2% (vol/vol) buffered formalin, and mixtures of ethanol/formalin/acetic acid were less satisfactory because they did not prevent diffusion of transferase from the cells. Higher concentrations of formalin destroyed the antigenicity of transferase, whereas glutaraldehyde (0.5-2.5%, vol/vol) introduced unsatisfactory levels of autofluorescence.

Enzyme Assay. Terminal deoxynucleotidyl transferase enzymatic activity in sonicated cell suspensions was assayed as described previously (12).

RESULTS

Distribution of transferase-positive cells in thymus sections

Frozen sections of rat, mouse, and calf thymus were exposed to serial 2-fold dilutions of rabbit antiterminal-transferase serum, and to the IgG and F(ab')2 fractions thereof, and were processed for indirect immunofluorescence. Although the pattern and intensity of specific fluorescence was not altered by fractionation of the antiterminal-transferase serum, there was a marked reduction in the level of nonspecific fluorescence in sections stained with the F(ab')2 antiterminal-transferase. A similar reduction in nonspecific staining occurred when the antiterminal-transferase preparations were absorbed with rat erythrocytes and viable lymph node cells or thymocytes. Inasmuch as these absorptions did not significantly affect antiterminal-transferase activity when tested by enzyme neutralization assay (13), they were performed routinely.

As shown in Fig. 1, rat cortical thymocytes fluoresced brightly with antiterminal-transferase (titer 1:80), whereas medullary thymocytes did not fluoresce detectably (titer <1:10). This was true for rat, mouse, and calf thymus sections. The ring-like pattern of fluorescence seen at the periphery of the lymphocyte could represent surface and/or cytoplasmic staining, because thymocytes have only a narrow rim of cytoplasm. There was no detectable nuclear staining in these preparations. It was our impression that thymocytes in the deepest part of the cortex (i.e., near the cortico-medullary junction) fluoresced less intensely than thymocytes elsewhere in the cortex, and that they were admixed with transferase-negative cells. Epithelial cells in the thymus medulla were transferase-negative. Epithelial cells in the thymus cortex were obscured by fluorescing thymocytes and could not be evaluated.

The specificity of the reaction of antiterminal-transferase with cortical thymocytes was confirmed by results of the following experiments: (i) preimmunization rabbit serum that had been absorbed with erythrocytes and lymph node cells did not react with cortical thymocytes; (ii) similarly absorbed antiterminal-transferase did not react with frozen sections of cervical, mesenteric, or bronchial lymph nodes; and (iii) reactivity of antiterminal-transferase for cortical thymocytes in sections was removed by absorption with purified calf transferase.

Cytological localization of transferase

F(ab')2 antiterminal-transferase (diluted 1:5) was incubated with suspensions of viable rat thymocytes and lymph node cells and developed for immunofluorescence. There was no detectable surface staining of either cell type. The titer of the antiterminal-transferase was not significantly reduced by absorption with viable thymocytes or lymph node cells, as determined by its ability to neutralize transferase activity (13) and to react with frozen sections of thymus.

F(ab')2 antiterminal-transferase did react with approximately 64% of total thymocytes in ethanol-fixed cell smears (Fig. 2a). The cytoplasm of positive cells fluoresced intensely, whereas the nucleus generally appeared to be negative. An occasional large thymocyte displayed very weak staining in the region of the nucleus. Ethanol-fixed lymph node cells showed neither cytoplasmic nor nuclear fluorescence (Fig. 2b).

Heterogeneity of cortical thymocytes

Suspensions of rat thymocytes were separated on 7-step Ficoll density gradients. The percentages of transferase-positive cells and cortical thymocytes were measured by immunofluorescence in each fraction (Fig. 3). Approximately 75% of total cortical thymocytes were transferase-positive. However, this value varied considerably in individual fractions of the gradient.
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**Fig. 2.** Smears of normal rat thymocytes and cervical lymph node cells, fixed in ethanol, exposed to F(ab')₂ antiterminal-transferase and developed with FITC-anti-rabbit IgG, ×525. Exposure 5 min (Kodak Tri-X Pan 35 mm film, ASA 400). (a) Thymocytes. There is intense cytoplasmic fluorescence of a majority of the cells in the smear (negative cells are indicated by arrows). There is no detectable nuclear staining beyond background levels seen in F(ab')₂ normal rabbit serum controls. (b) Lymph node cells. The cells appear as barely visible ghosts. There is no detectable nuclear or cytoplasmic fluorescence beyond background levels seen in controls (also compare with thymocytes). Bright particulate material is precipitated fluorescein conjugate.

For example, more than 95% of cortical thymocytes in fractions 5 and 6 were transferase-positive, whereas less than 10% of cortical thymocytes in fractions 1 and 2 were transferase-positive. Moreover, it was our impression that many of the transferase-positive thymocytes in fractions 3 and 4 fluoresced less intensely than the transferase-positive thymocytes in fractions 5–7. This suggested that heterogeneity exists among transferase-positive thymocytes with respect to transferase content.

In an attempt to document the latter point, transferase specific activity (measured enzymatically) was calculated as a function of the percent of transferase-positive cells in each fraction (determined by immunofluorescence). The results presented in Table 1 confirm our impression that transferase-positive cells in fractions 3 and 4 fluoresced less intensely with antiterminal-transferase than did cells in other fractions.

However, there was no apparent difference in intensity of fluorescence between transferase-positive cells in fractions 5 and 7, despite the marked differences in transferase specific activity (27.3 versus 9.3 transferase units/10⁶ transferase-positive cells, respectively).

**DISCUSSION**

The physiological function of terminal deoxynucleotidyl transferase is not known. The restriction of distribution of transferase to thymus and bone marrow has prompted speculation that it is involved in the functional differentiation of T cells, perhaps in immunological memory or diversity (1, 5, 17, 18). Whatever the merits of these proposals, it seems reasonable to anticipate that studies of the cellular and subcellular distribution of transferase may provide important insights both into the pathways of T cell development, and into the possible role(s) of transferase in this development. The availability of an antisera specific for transferase (13) promises to greatly expedite such studies.

In the present report we have directly confirmed our previous findings (11, 12) that transferase-positive thymocytes in the adult rat are restricted to the thymus cortex. We have extended these observations to include the thymus of the mouse and calf. However, not all cortical thymocytes were found to be transferase-positive, and not all transferase-positive cells appeared to contain equal amounts of enzymatically active transferase. This heterogeneity was demonstrated by analysis of suspensions of thymocytes separated on Ficoll density gradients. The results indicate that the majority of high- and low-density cortical thymocytes are transferase-negative, whereas almost all medium-density cortical thymocytes are transferase-positive (Fig. 3). Moreover, transferase-positive cells in low-density fractions...
appear to contain less enzyme than those in medium-density fractions, as evidenced by weak immunofluorescence with antiterminal-transferase and low transferase specific activity (compare fractions 3 and 5, Table 1). In contrast, some transferase-positive cells in the high density fraction may contain antigenically intact but enzymatically inactive enzyme. Such cells appeared to fluoresce as intensely with antiterminal-transferase as did medium-density thymocytes, but had only one-third the transferase specific activity (compare fractions 7 and 5, Table 1). Results of the present study indicate that transferase in cortical thymocytes is located predominantly within the cytoplasm. There was no detectable transferase on the surface of viable thymocytes. A nuclear pattern of fluorescence with antiterminal-transferase was only occasionally observed. This is in substantial agreement with the study of Chang (2), which showed that the bulk of transferase activity was present in the soluble (nonnuclear) fraction of disrupted thymocytes. However, that study also reported that approximately one-third of transferase activity in calf thymus was associated with the nuclear fraction, and that transferase activity was present in isolated rat thymocyte nuclei. The question of the exact intracellular distribution of transferase must therefore await more definitive immunocytological studies, preferably at the ultrastructural level.

Our inability to demonstrate transferase in medullary thymocytes or in lymph node by fluorescence is consistent with the findings of earlier biochemical studies (2, 11). Taken together, these results suggest that transferase does not exist in an enzymatically active or inactive form in recirculating T cells or in B cells. Moreover, it raises the possibility that transferase-positive cells and their descendants may not normally leave the thymus. Thus, it will be important to determine if medullary thymocytes and spleen-seeking thymocytes (19, 20) are derived from transferase-positive progenitors in thymus cortex. These considerations have been discussed elsewhere (12, 16).

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