Affinity purification and refined structural characterization of terminal deoxynucleotidyltransferase

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A total of 56 stable murine hybridoma monoclones that produce homogeneous antibodies against human or calf terminal deoxynucleotidyltransferase have been established. All of the antibodies exhibited specific binding to various M_r forms of terminal transferase and eight possessed neutralizing activity. Results are presented that permitted characterization of ten of these antibodies with respect to their immunoglobulin class, their recognition of calf or human terminal-transferase M_r species by immunoblotting techniques and their recognition of distinct antigenic sites. Terminal transferase was purified in a single step by using an immunoaffinity column constructed with a monoclonal antibody exhibiting a high binding affinity for the enzyme. Single monoclonal antibodies were also used to bind selectively to terminal-transferase antigen in tissue slices and individual cells.

INTRODUCTION

Terminal deoxynucleotidyltransferase is a template-independent DNA polymerase that will utilize any available dNTP in sequential addition to the 3' end of a DNA initiator (Bollum, 1962). Although the function of this enzyme *in vivo* is not well understood, the timing and tissue localization of its expression coupled with its catalytic characteristics *in vitro* suggest that it is relevant to the development of immune competence (Bollum, 1975). Terminal transferase has been proposed as a generator of somatic mutations in pre-lymphocytes to expand the coding diversity of DNA (Baltimore, 1974) and alternatively as an enzyme that alters the DNA of cortical thymocytes, thus marking them for destruction *in situ* (Rothenberg & Triglia, 1983). The existence of high levels of terminal transferase activity in the neoplastic counterparts of some pre-lymphocytes is an important differential diagnostic tool for human leukaemias (McCaffrey *et al.*, 1973; Greenwood *et al.*, 1977; Greaves, 1981; Coleman & Hutton, 1981). Although terminal transferase has been purified to apparent homogeneity from bovine, porcine, avian and human thymus as well as from human lymphoblasts, its primary structure is only beginning to be elucidated (Peterson *et al.*, 1984; Beach *et al.*, 1985). There exists a general consensus that the enzyme is synthesized as a single polypeptide of approx. molecular mass 62 kDa (Deibel & Coleman, 1979). This form of the protein is specifically fragmented to lower molecular-mass forms, including species of 55 kDa and 45 kDa and further fragmented to two subunits, approx. 30 kDa (β) and 12 kDa (α), both of which may be essential for catalytic activity (Bollum & Chang, 1981; Modak & Gillerman-Cox, 1982; Chang *et al.*, 1982). The proteinase(s) that catalyse(s) the fragmentation of terminal transferase appear(s) to exist in the highest concentration in thymocytes, whereas human lymphoblasts appear to be relatively deficient in this activity

(Deibel *et al.*, 1981). It is not yet clear whether the specific fragmentation of terminal transferase is of biological relevance or is an artefact introduced during the enzyme-purification steps.

We have prepared a series of 56 hybridomas that produce MAbs to terminal deoxynucleotidyltransferase to secure molecular reagents useful in the study of the biochemical structure of the enzyme and its manifestation in disease states. Here we describe the isolation and preliminary characterization of monoclonal hybridoma cell lines that produce antibodies directed against human or calf terminal deoxynucleotidyltransferase. The availability of this large battery of MAbs offered the possibility of radically improving the purification and immunodetection of terminal transferase. The procedures adapted in the present study resulted in (1) a shortened length of time for enzyme purification from 2½ weeks to 2 days, (2) a heretofore-unobtainable yield of high-molecular-mass forms of calf terminal deoxynucleotidyltransferase and (3) an excellent localization of terminal deoxynucleotidyltransferase antigen *in situ*.

A preliminary account of part of this work was presented at a meeting of the American Society of Biological Chemistry, held in San Francisco, CA, U.S.A., in June, 1983.

MATERIALS AND METHODS

Biochemical reagents for enzyme purification and assay were identical with those previously described (Deibel *et al.*, 1981). The standard enzyme assay for terminal transferase, as well as the definition of the unit of activity, were as reported previously (Coleman, 1977). Human-leukaemic-blast-cell and calf thymus terminal deoxynucleotidyltransferases were purified as previously described (Deibel & Coleman, 1980; Deibel *et al.*, 1981). BALB/c mice were obtained from Harlan Sprague-Dawley, Madison, WI, U.S.A. *Bordetella pertussis*

Abbreviations used: CPG, controlled pore glass; PMSF, phenylmethanesulphonyl fluoride; PEG, poly(ethylene glycol); phosphate-buffered saline (0.85 mM- Na_2HPO_4 /0.18 mM- NaH_2PO_4 /0.14 M- NaCl , pH 7.4); SDS, sodium dodecyl sulphate; e.l.i.s.a., enzyme-linked immunosorbent assay; e.i.a., solid-phase enzyme immunoassay; MAb, monoclonal antibody.

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vaccine was obtained from the Massachusetts Public Health Biologics Laboratory, Boston, MA, U.S.A. Dulbecco's modified Eagle's media, LoSM media, and media supplement NCTC-109 were purchased, respectively, from Flow Laboratories, McLearn, VA, U.S.A., Hybridoma Sciences, Atlanta, GA, U.S.A., and M.A. Bioproducts, Walkersville, MD, U.S.A. Tissue-culture plates (24- and 96-well, polystyrene and polyvinyl) were purchased from Costar, Cambridge, MA, U.S.A. Vector Laboratories (Burlingame, CA, U.S.A.) was the source of biotin-conjugated goat anti-mouse and anti-rabbit IgG, biotin-conjugated horseradish peroxidase and β -galactosidase, avidin D and Vectastain kits. Biotin-conjugated goat anti-mouse immunoglobulins (IgG, IgM) and goat anti-mouse immunoglobulins were obtained from Tago, Burlingame, CA, U.S.A., and Cappel Laboratories, Cochranville, PA, U.S.A., respectively. Nitrocellulose paper was purchased from Schleicher and Schuell, Keene, NH, U.S.A. PEG 1500 was obtained from BDH Chemicals, Poole, Dorset, U.K., and aminopterin, 2,6,10,14-tetramethylpentadecane and CNBr-activated Sepharose CL-4B were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Radioactive deoxynucleoside 5'-triphosphates were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). Unlabelled deoxynucleoside 5'-triphosphates were from Sigma. Controlled-pore glass (CPG/460 Glycophase G) was from Pierce Biochemicals (Rockford, IL, U.S.A.). Acrylamide and bisacrylamide were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Proteins used in M_r determinations were from Sigma and were further purified by gel filtration on Bio-Gel A-0.5 m.

Immunization protocol

BALB/c female mice were immunized with human-leukaemic-blast-cell or calf thymus terminal transferase (specific activity 20000–100000 units/mg of protein), given in multiple injections until antibody titre by enzyme-activity-neutralization assays was more than 1:200. The protein was adsorbed to $\text{Al}(\text{OH})_3$ gel, pelleted by centrifugation and resuspended in phosphate-buffered saline, pH 7.4, to which was added a *Bordetella pertussis* suspension.

Production and screening of hybridomas

Twelve mice immunized with calf terminal transferase and five mice immunized with human terminal transferase were used to produce hybridomas in ten sets of cell fusions (Oi & Herzenberg, 1980).

For e.l.i.s.a. screening of hybridomas for terminal-transferase antibody, terminal-transferase antigen was applied to 96-well polyvinyl plates and incubated overnight at 4 °C or for 4 h at room temperature, followed by a blocking step with 1% bovine serum albumin or chicken ovalbumin in borate/saline buffer (0.1 M-boric acid/0.025 M-sodium borate/0.075 M-NaCl), pH 8.5. After washing of plates, media from hybridoma cultures was added and incubated overnight at 4 °C. After phosphate-buffered saline washes, biotin-linked goat anti-mouse immunoglobulin was incubated in each well for 1 h at room temperature. Incubations of 30 min with 50 μl of avidin D (5 mg/ml in 0.1 M- NaHCO_3) and 30 min with 50 μl of biotin-linked β -galactosidase (5×10^5 units/ml in 10 mM-Hepes (pH 7.5)/1 mM- MgCl_2 /0.01% bovine serum albumin) followed phosphate-buffered

saline washes at each step. The substrate solution (5 mM-*o*-nitrophenyl β -galactopyranoside in 50 mM-sodium phosphate (pH 7.4)/8 mM- MgCl_2 /0.2% bovine serum albumin/0.2% 2-mercaptoethanol) was incubated for 30 min–1 h at room temperature. Absorbance was read at 420 nm.

Hybridoma cultures which were putative producers of terminal-transferase antibody by e.l.i.s.a. screening were cloned by limiting dilution in 96-well plates. Supernatants from clones found to be positive for terminal-transferase antibody by e.l.i.s.a. were examined by the immunoblot technique of Towbin *et al.* (1979).

For production of ascites fluid, BALB/c female mice were injected intraperitoneally with 2,6,10,14-tetramethylpentadecane. On day 7, 1×10^7 hybridoma cells suspended in 0.5 ml of phosphate-buffered saline or serum-free media were injected intraperitoneally. The ascites fluid was collected as appropriate, centrifuged at 20000 g for 15 min and stored.

MAbs were purified from media or ascites fluid by affinity-column chromatography with goat anti-mouse immunoglobulins (IgG + IgM + IgA) conjugated to CNBr-activated Sepharose 4B. Purified antibody was concentrated by ultrafiltration, washed with phosphate-buffered saline, and stored at -20 °C. Alternatively, IgGs were purified by Protein A-Sepharose affinity chromatography as described by Oi & Herzenberg (1980).

Enzyme-neutralization assays

Mouse sera diluted in phosphate-buffered saline or hybridoma media were added to an equal volume of calf thymus or human lymphoblast extract in 0.25 M-potassium phosphate buffer, pH 8.3, containing 0.25 unit of terminal-transferase activity. After an overnight incubation at 4 °C, the supernatants were assayed for enzyme activity as previously described (Deibel *et al.*, 1981).

Enzyme immunoassay

An enzyme immunoassay for terminal transferase was performed by the method of Coleman *et al.* (1985). Human terminal transferase (10 $\mu\text{g}/\text{ml}$) was incubated with goat anti-(calf terminal transferase)-coated polystyrene beads for 1.5 h at 30 °C. After a deionized-water wash step, a mixture of horseradish-peroxidase-conjugated rabbit anti-(calf terminal transferase) (0.4 $\mu\text{g}/\text{ml}$) and ascites fluid diluted 1:10 or 1:20 was added to the beads for the same incubation period. After a second water wash, the substrate solution (0.3% *o*-phenylenediamine dihydrochloride in 0.1 M-citrate/phosphate buffer, pH 5.5, containing 0.02% H_2O_2) was added. The enzymic reaction was allowed to proceed for 30 min at room temperature and the reaction was terminated by adding 1 ml of 5% (w/v) H_2SO_4 . Colour intensity was quantified at 492 nm.

MAbs were also substituted for polyclonal antisera in the enzyme immunoassay as a measure of binding affinity. Calf terminal transferase (100 ng/ml) was bound to the beads. Dilutions of ascites fluid were made in 10 mM-Tris buffer containing 30% calf serum and incubated with the beads for 1½ h at 30 °C. Peroxidase-conjugated goat anti-mouse IgG + IgM (0.2 $\mu\text{g}/\text{ml}$ in the same buffer) was then incubated with the beads under the same conditions after a wash step. Colour was developed as before.

Construction of immunoaffinity column

For construction of an immunoaffinity column, a single MAb (SFC 1) was used. The antibody was linked to CPG/460 Glycophase G through an avidin-biotin linkage. The first step in the procedure was to link avidin to the CPG. To dry CPG/460 Glycophase G was added 10 mM- NaIO_4 . The solution was placed under vacuum with stirring for 1 h. The activated CPG was washed with 250 vol. of water and 100 vol. of 0.1 M- K_2HPO_4 , pH 7.5. Avidin (Sigma Biochemicals) was diluted to 50 mg/ml with 0.1 M- K_2HPO_4 , pH 7.5, and combined with the washed CPG/460. The solution of 1% NaBH_4 was added and incubated at 4 °C for 15 min, followed by a second portion of NaBH_4 . The avidin-linked CPG was centrifuged at 1000 g for 10 min and resuspended in Tris-buffered saline.

The second step in the procedure was to biotinylate the MAb. The purified antibody was diluted to less than 10 mg of protein/ml in phosphate-buffered saline. A 0.25 vol. of 0.2 M- NaHCO_3 /0.15 M- NaCl , pH 8.8, was added, followed by the addition of *N*-hydroxysuccinimidobiotin (Sigma Biochemicals), in 0.1 M-dimethylformamide, to a concentration of 10 mg/ml. The solution was incubated at room temperature for 30 min and dialysed extensively against phosphate-buffered saline. The biotinylated antibody was then subjected to polyacrylamide-gel electrophoresis in SDS to monitor the increase in the M_r of the antibody.

Finally, the avidin-linked CPG/460 and biotinylated antibody were gently mixed by rotation at 4 °C for 4 h. The extent of binding of the antibody to the CPG/460 was monitored by checking the effluent from the column material for protein (using the A_{280}/A_{260} ratio).

Fig. 1. Characterization of MAbs by immunoblot analysis

(a) Terminal transferase analysed by SDS/polyacrylamide-gel electrophoresis and stained with Commassie Blue: M, standards; 1, calf enzyme; 2, human enzyme. (b) Immunoblot of terminal transferase with polyclonal rabbit anti-calf terminal transferase): M, standards stained with Amino Black; 1, calf enzyme; 2, human enzyme. (c) Immunoblot of calf terminal transferase with MAbs: 1, SFH 4; 2, SFC 44; 3, SFC 37; 4, SFC 16; 5, SFC 1; 6, SFC 6; 7, SFC 32; 8, SFC 36; 9, SFC 22; 10, SFC 3.

Table 1. Characteristics of selected MAbs directed against human or calf terminal transferase

Each MAb was tested for binding to terminal transferase species by immunoblot analysis. A '+' indicates that a protein with the molecular mass indicated was detected, whereas '-' indicates that no binding was observed. Each MAb was also tested for neutralization of terminal transferase enzymic activity. In the right-hand column, '+' indicates neutralization was observed, whereas '-' indicates no inhibition of activity.

	Antigen						Immuno- globulin	Neutral- ization	
	Calf					Human			
	62 kDa	55 kDa	30 kDa	26 kDa	12 kDa	62 kDa			45 kDa
SFC 4	—	+	+	+	—	+	+	IgM	—
SFC 44	+	+	+	+	—	—	—	IgG	+
SFC 37	+	+	+	+	—	—	—	IgG	+
SFC 16	+	+	+	+	—	—	—	IgG	+
SFC 1	+	+	+	+	—	+	+	IgG	+
SFC 6	+	+	+	+	—	—	—	IgG	—
SFC 32	+	+	+	+	—	—	—	IgM	—
SFC 36	—	+	+	+	—	—	—	IgG	+
SFC 22	—	+	—	—	—	+	+	IgG	—
SFC 3	—	+	—	—	—	+	+	IgM	—

Immunoperoxidase staining of terminal transferase

Thymus cells, thymus sections and blood smears were fixed in 100% methanol for 30 min at 4 °C. The slides were then incubated 3 min with 3% H₂O₂ in phosphate-buffered saline, washed three times with phosphate-buffered saline, and blocked for 15 min with 5% goat serum in phosphate-buffered saline. After further phosphate-buffered saline washes, individual monoclonal antibodies (SFC 1, SFC 16 or SFC 37) or polyclonal rabbit antisera, diluted in phosphate-buffered saline were incubated with the slides for 1–2 h at room temperature. Slides were washed three times with phosphate-buffered saline and incubated for 45 min with biotinylated goat anti-mouse or anti-rabbit immunoglobulin diluted in phosphate-buffered saline. After washes with phosphate-buffered saline, the slides were incubated 20 min with Vectastain solution (Vector Laboratories, Burlingame, CA, U.S.A.; Avidin D with biotinylated horseradish peroxidase diluted with 1% horse serum in phosphate-buffered saline). After several washes with phosphate-buffered saline, the slides were immersed in substrate solution (0.5 mg of 3,3'-diaminobenzidine/0.3% H₂O₂/0.02% CoCl₂/ml of phosphate-buffered saline). After 20 min of colour development, the slides were washed three times in phosphate-buffered saline and sealed with mounting fluid.

RESULTS

Characterization of antibodies

Initial attempts to develop hybridoma cell lines producing antibody against terminal transferase indicated that it was essential to achieve high titres of circulating antibody in the immunized mice before splenectomy. Nearly all of the first 20 MAbs directed against calf terminal transferase were μ -heavy-chain producers. Only three synthesized γ -heavy chains. When MAbs obtained from mice immunized with human terminal transferase were examined, all were found to be μ -chain producers. These hybridomas were obtained from mice immunized

over a 3–4-month period. By extending the immunization protocol to 10–12 months, the percentage of the more useful IgG producers was increased to more than 90%.

The pattern of binding of individual antibodies to the spectrum of terminal-transferase antigens displayed by SDS/polyacrylamide-gel electrophoresis followed by immunoblot analyses are presented in Fig. 1. The interpretation of these binding patterns is simplified by inspection of the SDS/polyacrylamide-gel patterns of purified calf thymus (lane 1) and human lymphoblast (lane 2) terminal transferase stained with Coomassie Blue (Fig. 1a). These patterns are characteristic and distinguishable. The calf terminal transferase has predominant protein bands at 30 kDa (β -subunit) and 12 kDa (α -subunit) and a range of higher-molecular-mass forms between 45 kDa and 62 kDa. The predominant protein species in the human terminal-transferase preparations are at approx. 62 and at 45 kDa. Immunoblot analysis of these antigen preparations with polyclonal rabbit anti-(calf terminal transferase) is illustrated in Fig. 1(b). Note that the immunoblot and stained-gel patterns are virtually identical. When individual MAbs were utilized in the immunoblotting experiments with calf terminal transferase as antigen, the staining patterns shown in Fig. 1(c) were observed. Each of the ten lanes represents an individual MAb. The patterns illustrated are representative of all those obtained with antibody derived from 56 monoclones. The antibody in lane 1 (SFH 1) was derived from mice immunized with human lymphoblast terminal transferase. This MAb cross-reacted with calf antigen, recognizing 55 kDa, 30 kDa (β -subunit) and 26 kDa (β' -subunit, a common breakdown product of the β -subunit) species. The next seven antibodies (lanes 2–8) showed almost identical staining, except that antibodies in lanes 2–6 also bound relatively strongly to 62 kDa terminal transferase. Antibodies in lanes 1 and 7–10 appear to have bound only weakly to the 62 kDa protein. Two antibodies in the group (SFC 22 and SFC 3 in lanes 9 and 10) detected the 55 kDa terminal transferase and did not appear to bind at detectable levels to lower- M_r forms of the protein. Unlike the polyclonal antisera, none

Table 2. Competition of polyclonal antibody in enzyme immunoassay by MAb

An enzyme immunoassay was used to estimate the minimum number of distinct immunological epitopes of terminal transferase. Each MAb was tested for competition in polyclonal antibody binding to terminal transferase as described in the Results section.

MAb	Competition (% of control-immunoassay value $\pm$ S.D. (n))	Ascites dilution effective in immunoassay
SP2/0	98.4 $\pm$ 3.6 (5)	0
SFC 1	67.7 $\pm$ 1.1 (4)	> 78 000
SFC 37	66.3 $\pm$ 3.9 (5)	> 78 000
SFC 16	39.8 $\pm$ 1.8 (5)	3 125
SFC 36	77.7 $\pm$ 13.5 (3)	ND
SFC 32	71.2 $\pm$ 4.1 (2)	ND
SFC 6	98.9 $\pm$ 2.0 (3)	ND
SFH 4	96.5 (2)	0
SFC 44	105 (3)	0
SFC 22	98 (4)	0
SFC 3	97 (4)	0
SFC 1 + SFC 16	27.9 $\pm$ 3.8 (4)	
SFC 37 + SFC 16	29.2 $\pm$ 4.0 (4)	
SFC 37 + SFC 1	61.2 $\pm$ 3.7 (3)	
SFC 37 + SFC 36	57.1 (2)	
SFC 37 + SFC 6	55.3 $\pm$ 3.7 (3)	
SFC 37 + SFH 4	53.5 (2)	
SFC 1 + SFC 36	70.0 (2)	
SFC 1 + SFC 6	72.1 (1)	
SFC 1 + SFH 4	68.2 (1)	
SFC 37 + SFC 32	58.0 (1)	

*ND, not determined.

of the MAbs was shown to react with the 12 kDa subunit (α) of terminal transferase. When the human enzyme was used as antigen, only four of these antibodies were shown to react by immunoblot analysis (SFH 4, SFC 22, SFC 1, and SFC 3) (results not shown). Thus 40% of the MAbs in this group reacted with calf and human species by this analysis. A summary of the immunoblotting data and immunoglobulin classification of the antibodies is given in Table 1.

To estimate the minimum number of distinct immunological epitopes of terminal transferase which were physically distinguishable in the group of antibodies we isolated, ten antibodies were tested in an e.i.a. (Coleman *et al.* 1985). The solid-phase immunoassay consisted of primary-antibody-coated polystyrene beads [goat anti-(calf terminal transferase)] and a secondary antibody of rabbit anti-(calf terminal transferase) conjugated to horseradish peroxidase. To detect terminal-transferase antigen the enzyme preparation was incubated with primary-antibody-bound beads. After washing, the secondary antibody was incubated with the bead-linked antibody-enzyme complex. A heterologous system (rabbit and goat antisera) was used to maximize the sensitivity for detecting different antigenic sites on the protein. Quantification of antigen was effected by adding a colorimetric peroxidase substrate to the protein complex. The assessment of antigenic sites represented by MAbs was carried out by adding ascites fluid produced from individual hybridomas to a secondary polyclonal-antibody conjugate. A titration of the MAb was performed to achieve the appropriate dilutions. If the added MAb inhibited colour development after addition of the peroxidase substrate, we postulated that com-

petition by MAb for a polyclonal-antibody binding site had occurred.

The data from the single competition experiments are given in Table 2. Ascites fluid from the fusion partner, non-Ig-producing hybridomas and hybridomas producing anti-(human adenosine deaminase) MAbs were included as controls. None interfered with the solid-phase immunoassay (results not shown). In contrast, two MAbs, SFC 1 and SFC 37, reduced colour development to less than 70% of that of the control. A third MAb, SFC 16, decreased polyclonal antibody binding to 40% of control. Other single MAbs, SFC 36 and SFC 32, provided 20% and 29% inhibition of colour development. On the other hand, SFC 6, SFC 44, SFC 22, SFC 3 and SFH 4 had no effect. These data suggest that the antigenic sites recognized by the goat anti-calf polyclonal antibody in the configuration limits imposed by the e.i.a. were not represented among these MAbs.

In the next series of experiments we sought to group the MAbs with respect to epitope recognition. These experiments were based on the assumption that, if the MAbs recognized different epitopes, the competition with the polyclonal antibody would be additive. Therefore we modified the experimental protocol, described above, by allowing the polyclonal-antibody binding to complete with that of a mixture of two MAbs.

In the lower portion of Table 2 are presented results from pairwise mixtures of MAbs. Hybridomas SFC 1 and SFC 37 appear to bind the same epitope, since they both compete with the polyclonal antibody when used alone, do not give increased inhibition when mixed, and complement the inhibition of SFC 16 similarly when mixed with that antibody. That they are somewhat unlike

Table 3. Purification of calf terminal transferase by MAb affinity column

Fraction	Volume (ml)	Protein (mg)	Specific activity* (units/mg)	Total activity (units)	Yield (%)	Purification factor
I. Crude extract	940	17388	14.5	251638	100	—
II. Immunoaffinity	0.95	0.749	171943	122346	49	11858

* Represents polymerization of Mg^{2+} -dATP on to a synthetic initiator, $p(dA)_{50}$. One unit of enzyme activity is defined as 1 nmol of radioactive deoxynucleotide incorporated/h.

in their binding of terminal transferase is also evident, since SFC 37 in combination with SFC 36, SFC 32, SFC 6 and SFC 4 gives increased competition with the polyclonal antibody, whereas the same pairings with SFC 1 give no additional effects. It is possible that the binding of SFC 37 antibody to terminal transferase may alter the antigenic conformation such that the SFC 6 and SFH 4 MAbs now have binding sites available, since neither alone had any effect on the e.i.a. Thus at least four groups of MAbs are defined with respect to epitope recognition: (1) SFC 1 and SFC37, (2) SFC 16, (3) SFC 36, SFC 6, SFC 32 and (4) SFC 44, SFC 22, SFC 3 and SFH 4. Since the latter group of five antibodies did not compete with polyclonal antibody, they represent epitopes not recognized by the polyclonal antibody and therefore cannot be classified in this experiment.

These MAbs were further characterized with respect to relative binding affinities and neutralization of terminal-transferase activity. The MAbs were screened in a modification of the enzyme immunoassay described above. The primary polyclonal antibody bound to the polystyrene bead was used to bind soluble terminal transferase and the secondary antibody was a single MAb. A peroxidase-conjugated-goat anti-mouse IgG + IgM was used to provide the colour development. The third column of Table 2 shows the results of these experiments. There was a direct correlation between the ability of a MAb to compete in the preceding experiment and its apparent binding affinity in this experiment. Five of the MAbs neutralized terminal-transferase activity in solution. Unconcentrated media from these hybridoma cultures, SFC 16, SFC 1, SFC 36, SFC 37 and SFC 44, demonstrated 25–67% inhibition of terminal-transferase catalytic activity. Since the MAbs that neutralized recognized four different antigenic sites (Table 1), it appears that the catalytic site can be affected by binding to four different epitopes.

Purification of terminal transferase with a MAb column

Frozen calf thymus (460 g) was broken into small pieces with a meat grinder. The thymus tissue was extracted in 2 vol. of 0.25 M-potassium phosphate buffer, pH 7.4, containing 1 mM-2-mercaptoethanol. Some enzyme preparations contained the following proteinase inhibitors: 2 mM-PMSF, 5 mM-benzamide, ovomucoid (0.25 mg/ml), aprotinin (50 μ g/ml). The suspension was stirred at 4 °C for 60 min and centrifuged at 25000 *g* for 60 min (Sorvall, GSA) to yield fraction I (crude supernatant). Fraction I was incubated overnight (gentle rotation) at 4 °C with the MAb linked to CPG/460 (avidin-biotin linkage). Approx. 7 ml of column material were used for 0.5 kg of calf tissue. More than 90% of

enzyme activity in the crude extract bound to the column material. The suspension was equilibrated to room temperature, the supernatant was decanted and the gel material was washed three times with 50 ml portions of 0.25 M-potassium phosphate, pH 7.4. After these steps, the gel was transferred to a 1 cm $\times$ 10 cm column and washed with 20 ml of 0.2 M-KCl in 0.25 M-potassium phosphate, pH 7.4. To elute non-specifically bound proteins, the column was washed sequentially with 40 ml of 2.0 M-KCl in 0.25 M-potassium phosphate, pH 7.4, and 40 ml of 2.0 M-KCl in 0.025 M-potassium phosphate, pH 7.4. The final wash was with 20 ml of 0.05% Triton X-100 in 0.025 M-potassium phosphate, pH 7.4. Terminal-transferase activity was eluted with 10 ml of 2.0 M-MgCl₂, immediately concentrated by ultrafiltration (PM-10 membrane) and dialysed by ultrafiltration into Tris buffered saline, pH 8.0. The concentrate (fraction II) was assayed for enzyme activity and protein. The results of purification are shown in Table 3.

The specific activity of terminal transferase in the purified fraction was 171943 units/mg of protein, indicating that fraction II had a specific activity close to that which we have previously obtained for enzyme preparations shown to be essentially homogeneous by non-denaturing polyacrylamide-gel electrophoresis (Deibel & Coleman, 1979). Analysis of fraction II by SDS/polyacrylamide-gel electrophoresis showed that high-molecular-mass terminal transferase was the major species of purified enzyme. Inspection of the SDS/polyacrylamide-gel stained with Coomassie Blue (Fig. 2a, lane 2) reveals the typical high-molecular-mass (45–62 kDa) terminal-transferase protein bands in fraction II. Only small amounts of the lower-molecular-mass species (α and β -subunits) are observed in fraction II. An immunoblot analysis of fraction II and purified calf thymus terminal transferase is shown in Fig. 2(a) (lane 3). The protein bands observed in the stained gel also bind anti-(terminal transferase) antibody (with the exception of the β -subunit) and indicate that virtually all of the proteins represented are authentic terminal transferase. The predominantly high-molecular-mass species are contrasted with terminal-transferase protein obtained by conventional purification procedures (Fig. 2b, lane 1). With this preparation, the predominant protein bands are the α -(12 kDa) and β -(30 kDa) subunits of low-molecular-mass terminal transferase. Their antibody-binding capacity is shown in Fig. 2(b) (lane 2).

Immunohistochemical localization of terminal transferase with MAbs

Each of the ten MAbs characterized was tested with an immunoperoxidase procedure to localize terminal trans-

Fig. 2. Polyacrylamide-gel electrophoresis and immunoblot of calf terminal transferase purified by (a) an immunoaffinity column and (b) a conventional purification scheme

This Figure shows a comparison of calf terminal transferase obtained by two separate purification schemes. (a) Lane 1, molecular-mass marker proteins, stained with Coomassie Blue; lane 2, fraction II calf terminal-transferase proteins (specific activity 171 000 units/mg) stained with Coomassie Blue; lane 3, immunoblot of fraction II with rabbit anti-(calf terminal transferase) as antibody. (b) Lane 1, preparation of pure calf terminal transferase (specific activity 163 000 units/mg) purified by a conventional scheme (Deibel & Coleman, 1980); lane 2, immunoblot of pure calf terminal transferase with rabbit anti-(calf terminal transferase) as antibody.

Fig. 3. Immunoperoxidase staining of thymus and leukaemic cells with MAb

Tissue sections or cells were fixed and allowed to react with antibodies as described in the Materials and methods section. (a) Human thymus section stained with MAb (magnification $\times 12.8$); (b) cortex/medulla interface on a human thymus touch preparation (magnification $\times 32$); (c) terminal-transferase-positive human lymphoblast (arrow) (magnification $\times 320$); (d) terminal-transferase-negative cell (arrow) (magnification $\times 320$).

ferase within cells and tissues. Three of the ten (SFC1, SFC 16 and SFC 37) were individually useful in binding and localizing terminal-transferase antigen. In Fig. 3 are examples of the staining patterns that were observed with the MABs. When human thymus tissue slices were stained with antibody SFC 1, a sharp distinction between cortical and medullary areas of the thymus was observed (Fig. 3a). A higher magnification of this thymus section illustrates that the staining is confined to nuclei of cortical thymocytes, with virtually no staining of medullary areas of the tissue (Fig. 3b). The pattern of nuclear staining in terminal-transferase-containing cells is clearly seen in the photomicrograph in the field shown in Fig. 3(c), in which a human leukaemic lymphoblast was stained with monoclonal antibody SFC 1 by using the peroxidase procedure. The typical indentation of the nucleus with an unstained cytoplasm is apparent. Fig. 3(d) shows a polymorphonuclear cell that has been allowed to react with the MAB. This non-terminal-transferase-containing cell did not bind any of the antibody, an observation that is important because immunocytochemical procedures using polyclonal antibody have been plagued with non-specific binding to polymorphonuclear cells (Cibull *et al.* 1981).

DISCUSSION

Monospecific polyclonal antibodies against both human and calf terminal deoxynucleotidyltransferase have been useful in determining the intracellular localization of the enzyme (Bollum, 1979) and in confirming that low-molecular-mass forms are generated from high-molecular-mass species (Chang *et al.* 1982). Previous work on terminal transferase isolated from avian and mammalian species indicated that the protein was highly conserved during evolution (Chang *et al.* 1982). Access to a variety of MABs directed against various portions of terminal transferase as well as against terminal transferase from two mammalian species (calf and human) will allow one to pin-point structural differences between the forms of this protein from a variety of sources.

In the present paper we report the successful establishment of 56 stable murine hybridoma lines and the characterization of ten representative monoclonal antibodies that produce homogeneous IgG or IgM molecules that recognized various segments of the calf or human terminal-transferase molecule. The highest molecular-mass species detected was 62 kDa. The observation that all variant molecular-mass forms react with a single MAB demonstrates that these forms possess a common antigenic site and supports the hypothesis that they are all generated from a common precursor. The 30 kDa subunit was extremely antigenic in the mouse, but the 12 kDa enzyme subunit was not, since none of the 56 monoclonal antibodies isolated secreted antibody that bound this α -subunit tightly enough to be detected in the immunoblot analyses. Also apparent from the immunoblotting experiments was an indication that the MABs could differentiate between human and calf terminal transferase, since six out of nine anti-calf antibodies failed to bind to human terminal transferase.

The activity-neutralization experiments combined with the antibody-competition studies demonstrated that a minimum of four separate antigenic sites were represented among the antibodies. The enzyme immunoassay we used

to distinguish antigenic sites has physical limitations, since the antibody-bound antigen presents only a few possible binding sites in the assay. The only sites detected will be those present in the goat antiserum against which MABs compete. A modification of the e.i.a. in which the secondary antibody was replaced with MAB demonstrated that apparent binding affinities of the antibodies varied by greater than five orders of magnitude.

Selected MABs with the highest binding affinities were used for construction of an immunoaffinity column for terminal transferase as well as for immunocytochemical localization of terminal-transferase antigen.

The intracellular localization of thymic terminal transferase has been established by subcellular-fractionation studies (Chang, 1971) and by immunofluorescence detection of the enzyme with polyclonal antibodies (Gregoire *et al.* 1977; Cibull *et al.* 1982). In certain cases, terminal transferase has been found in the cytoplasm (Goldschneider *et al.* 1977) as well as in the nucleus (Janossy *et al.* 1979, 1980); however, it has been suggested that cytoplasmic terminal transferase may be functionally inactive *in vivo* (Gregoire *et al.* 1977). By using the MAB SFC 1, we observed nuclear staining in both thymus and leukaemic lymphoblasts. The staining patterns obtained with tissue sections and blood smears were well defined and lacked the apparent 'artificial' staining of polymorphonuclear cells that has been observed with polyclonal antibodies. These MABs will therefore be useful in expanding the battery of immunocytochemical reagents applicable for the differential diagnosis of adult and childhood leukaemia and lymphoma and for defining the localization of terminal-transferase antigen during differentiation of lymphocytes and after neoplastic transformation.

The affinity purification reported herein included extraction of the enzyme in relatively-high-ionic-strength buffer and binding at high ionic strength to the MAB column. Elution of the column with $MgCl_2$ resulted in an overall purification of approx. 12000-fold and a yield of 50% of starting enzyme activity. On the basis of the high specific activity and the SDS/polyacrylamide-gel electrophoresis profile, the enzyme appeared to be near homogeneous. A striking difference between the molecular-mass species present in protein preparations obtained by immunoaffinity purification and those purified by conventional schemes was observed. The procedure detailed here preferentially yielded high-molecular-mass calf terminal transferase rather than the two-subunit form of the enzyme that had been previously obtained. Although some breakdown of the enzyme occurred even in the presence of a number of proteinase inhibitors, this procedure will clearly be useful, not only for rapid enzyme purification, but also for generation of high-molecular-mass calf thymus terminal transferase. The latter had heretofore been almost impossible to obtain in reasonable yield, even when a variety of proteinase inhibitors were used during conventional purification. The ratio of tissue (~0.5 kg) to column volume (7 ml) chosen for purification did not fully saturate the binding capacity of the MAB column. However, the volume constraint imposed by the small amount of column material compared with large extract volume (1 litre) rendered binding any additional enzyme from crude extract impractical. A fivefold expansion in the volume of column material should make purification of 5–10 mg of protein in a single step feasible.

The immunoaffinity column constructed has two advantages over the columns commonly employed. According to the procedure we devised, the use of CPG as the matrix material yielded a gel with excellent longevity and flow properties; the avidin-biotin linking procedure permitted the use of both IgM and IgG MAbs.

Kinetic studies of terminal transferase indicate that the extra protein sequences found in the high-molecular-mass enzyme influence initiator binding. These studies indirectly suggested that these protein sequences may play an auxiliary role in positioning the DNA initiator substrate (Deibel & Coleman, 1980) so it can interact optimally with the monomer substrate to produce catalysis. All previous physical-chemical studies have utilized the low-molecular-mass enzyme. The purification procedure we report here will allow direct physical studies of oligonucleotide binding to be extended to high-molecular-mass enzyme.

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