DNA-DEPENDENT DNA AND RNA POLYMERASES AND tRNA-METHYL-TRANSFERASES IN HUMAN LEUKEMIA AND DIFFERENTIATING FRIEND VIRUS LEUKEMIA CELLS*

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DNA-dependent DNA and RNA polymerases play a central role in the process of gene replication, gene expression, gene amplification, and also gene regulation. At present, RNA-dependent DNA polymerase (reverse transcriptase) has attracted particular interest in connection with the problem of a viral etiology of human leukemia. Considering the importance of DNA-dependent RNA polymerases for transcription and replication, these enzymes also must be important for the process of malignant transformation. Evidence for direct participation of RNA synthesis in DNA replication has been reported for phages, bacteria, and, recently, for eucaryotic cells (1). RNA polymerases have been highly purified from bacteria. It has been shown, that the complete or so-called holoenzyme could be separated into several subunits with defined functional properties (2). Holoenzyme consists of the sigma factor and the core enzyme with its subunits alpha 1, alpha 2, beta, beta′, and a number of smaller factors called psi, kappa, rho. Sigma catalyses the specific initiation of RNA synthesis on the promoter site of DNA. Core enzyme is able to synthesize RNA but lacks the ability for specific initiation. Beta subunits are responsible for the binding to DNA, kappa for the arrest, and rho for the termination of transcription (3). ZILLIG and coworkers demonstrated that, after infection with bacteriophage T_4, E. coli RNA polymerase is altered in two consecutive steps. The purified enzyme from phage-infected cells does not contain sigma, and exhibits structural changes in all subunits of core enzyme (4, 5, 6).

Polymerases from eucaryotic cells are extremely unstable enzymes complicating purification and analysis. Thus very little is known about properties and function of mammalian RNA polymerases up to now. DNA-dependent RNA polymerases are differentiated according to their sensitiveness to the mushroom toxin alpha-amanitin and their preference of native or denatured DNA templates (Table 1). The first group of polymerases, termed A, is not inhibited by alpha-amanitin and prefers native DNA templates, while the second group of RNA polymerases, called B, is completely inhibited by low doses of alpha-amanitin, and prefers denatured DNA templates. In

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Enzyme	A			B			
Characteristic	A I	A II	A III	B I	B II	C	"mit"
Subcellular localization	nucleolus	nucleolus	nucleoplasm	nucleoplasm	nucleoplasm	cytoplasm	mitochondria
Size	16 S						
Molecular weight	500 000			500 000	500 000	500 000	
Function	rRNA synth.			mRNA synth.			mitRNA synth.
Template preference ratio nat./denat. DNA	20	16	1	0.5		0.74	0.57
Divalent cations ration Mn/Mg	1.1	0.7	3	5		3.5	0.55
Optimum ionic strength							
$(NH_4)_2SO_4$	40 mM	55 mM	40–200 mM	100 mM			10 mM
KCl	200 mM	200 mM					
Optimum pH	8.0	8.0		8.7			
Elution from DEAE cellulose							
$(NH_4)_2SO_4$	0.17 M		0.4 M	0.2 M	0.35 M		0.25 M
Ionic strength	0.2			0.3		0.22	
Elution from phosphocellulose							
NH_4CL	0.45 M			0.4 M		0.35 M	
KCl	0.50 M	0.65 M					
+ 0.1 µg/ml α-amanitin	100 %			0 %		75 %	
+ stimulating factor "B"	115 %			270 %		109 %	

Table 1: Reported properties of DNA dependent RNA polymerases from mammalian cells.

addition, RNA polymerase is found in a deoxyribonucleoprotein complex, demanding high ionic strength for optimum transcription. Besides these polymerase activities, located in the nucleus, a cytoplasmic enzyme with intermediate sensitivity to alpha-amanitin (7) and a mitochondrial RNA polymerase (8) have been described. RNA polymerase A synthesizes rRNA und tRNA precursors, RNA polymerase B catalyzes the transcription of mRNA precursors (heterogenous nuclear RNA). Polymerase A has been fractionated into two large subunits. Three large subunits have been described for the B enzymes. In addition, each enzyme molecule possesses several subunits of lower molecular weight (9). In contrast to the E. coli enzyme, no defined functional properties can be associated with the subunits of these eucaryotic enzymes, so far.

In the first stage of our investigations, RNA polymerases A and B were characterized in nuclear fractions from isolated lymphocytes, granulocytes, or total leukocytes. Cells were isolated from 10–40 ml of venous blood by accelerated sedimentation

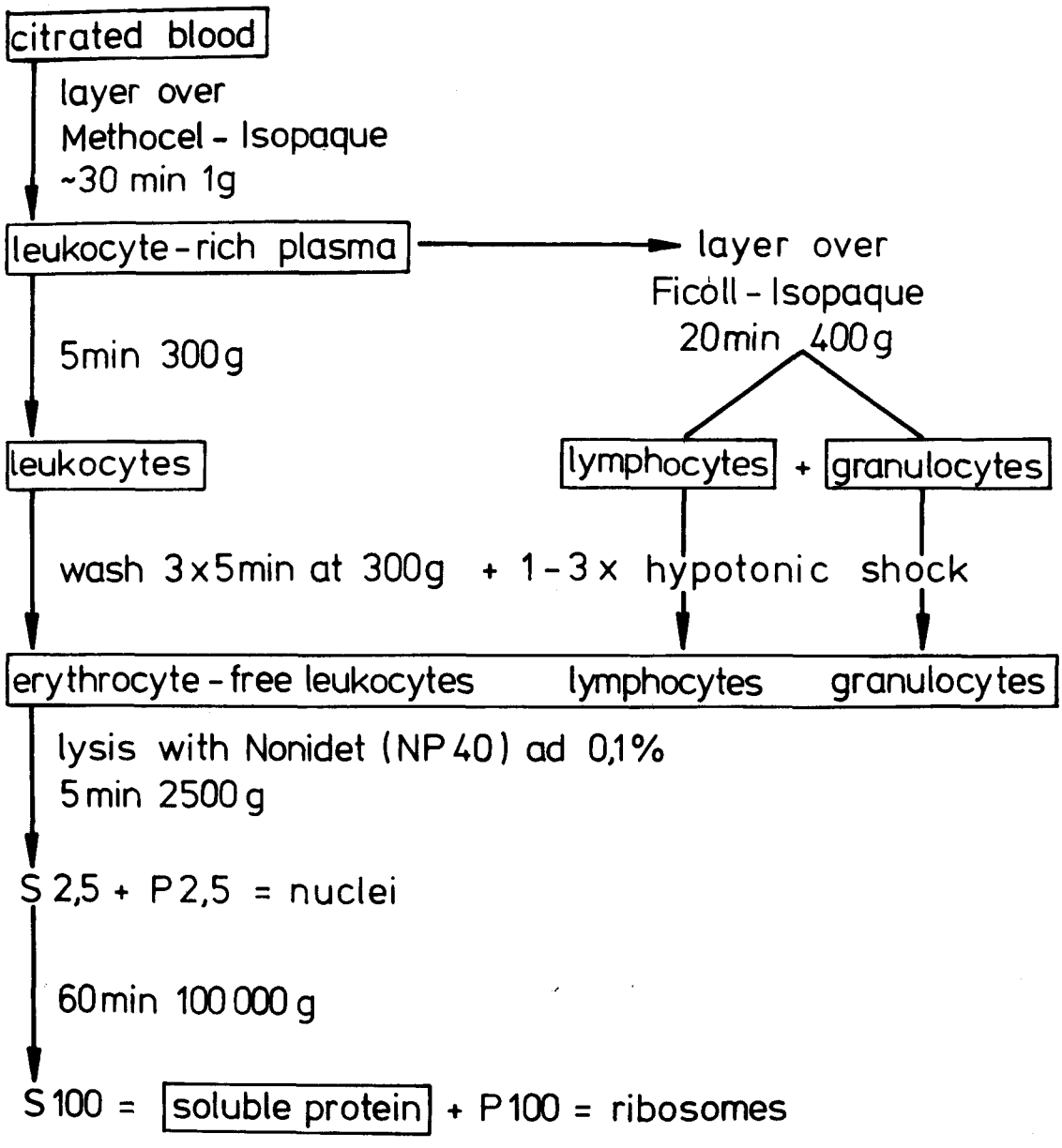

Fig. 1: Isolation of leukocytes, separation into lymphocytes and granulocytes, and preparation of nuclei and 100 000 g supernatant.

The method is a modification of the procedure of Böyum*). 10 ml citrated blood (3.8 % Na-citrate 1 : 5) are layered on top of 5 ml of a Methocel-Isopaque mixture (16 parts Methocel, Fluka, 2 % in aqueous solution mixed with 10 parts Isopaque = Ronpacon 75 %, Cilag, sterilized 30 min at 120° C). The leukocyte-rich plasma was diluted to 15 ml by isotonic buffer (IB: 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 1 mM K-EDTA, 90 mM NaCl) and layered over 7 ml of a Ficoll-Isopaque mixture (24 parts Ficoll, Pharmacia Uppsala, 9 % in aqueous solution, 10 parts Isopaque = Ronpacon 75 %, sterilized 30 min at 120 °C). After 20 min centrifugation at 400 xg the lymphocytes banded at the interface, the granulocytes together with a few contaminating erythrocytes precipitated to the bottom of the tube. Cells were washed with IB (3 x 5 min 300 g) and erythrocytes lysed by 1—3 hypotonic shocks (by addition of 2 ml bidistilled water and after 20 sec 2 ml 1.8 % NaCl). Cells were resuspended in IB to a concentration of 50 000/µl and lysed by addition of 0.1 % Nonidet (NP 40, Shell). Nuclei were immediately spun off (5 min 2500 g). The supernatant was centrifuged 60 min at 100 000 g.

*)Böyum, A.: Separation of leukocytes from blood and bone marrow; Scand. J. Clin. Lab. Invest. *21*, Supp. 97 (1968).

(Fig. 1). The resulting leukocyte rich plasma was separated into lymphocytes and granulocytes on a Ficoll-Ronpacon gradient. Leukocytes were disrupted by sonication or by addition of 0.1 % NP-40, and nuclei spun off at 2500 x g (5 min). The resulting pellet, consisting of nuclear debris, was resuspended, homogenized, and used for investigation of RNA polymerase activity (fraction II P. For details of methods cf. 10, 11). Polymerase activities were differentiated by appropiate incubation conditions: RNA polymerase A was tested in the presence of 10 mM $MgCl_2$, 50 mM ammonium sulphate (AmS), native DNA templates and 0.02 µg alpha-amanitin, RNA polymerase B in the presence of 2.5 mM $MnCl_2$, 100 mM AmS, denatured DNA templates, chromatin-bound RNA polymerase in the presence of 2.5 mM $MnCl_2$, 400 mM AmS, being neglegibly stimulated by addition of DNA templates.

The nuclear fraction, obtained as described, contains a high amount of endogenous DNA, nevertheless RNA polymerases A and B are highly dependent on exogenous template DNA (11). This could be caused by a very high degree of repression of endogenous chromatin DNA. No significant qualitative differences have been found between RNA polymerases A, B, and the chromatin bound enzyme from normal and leukemic leukocytes, regarding the efficiency of different DNA templates (Tab. 2), the dependency on divalent cations and ionic strength (Tab. 3), and the inhibition by

RNPASE	A	A	B	B
DIAGNOSIS	NL	CLL	NL	CLL
CONDITIONS	%	%	%	%
complete	100	100	100	100
- DNA	15	56	13	6
- denat., + nat. DNA	100	100	23	15
- nat., + denat. DNA	56	45	100	100
TQ	0.56	0.45	4.2	5.8

TQ = quotient of transcription of denatured versus native DNA template

Table 2: Influence of DNA template on DNA-dependent RNA polymerases in isolated nuclei from human peripheral blood leukocytes.

NL = normal leukocytes; CLL = chronic lymphatic leukemia; RNA polymerase activity was measured by the incorporation of ^{3}H-UMP into acid precipitable polynucleotides in the presence of the following components: ^{3}H-UTP (spec. act. 10 Ci/mmole) 1 µM; ATP, CTP, and GTP 1 mM; 20 µg DNA (native for RNA polymerase A, denatured for RNA polymerase B); 200 µg protein of fraction II P; Tris-HCl pH 8.5 200 mM; 2-mercaptoethanol 8 mM; glycerol 9 % (v/v); and 0.3 mM K-EDTA in a final volume of 100 µl (divalent cations and ammonium sulphate concentrations as described in the text).

RNA Polymerase	A	A	B	B
Diagnosis	NL	CLL	NL	CLL
Conditions:				
complete	100 %	100 %	100 %	100 %
- $MgCl_2$, + $MnCl_2$	206 %	198 %	100 %	100 %
- $MnCl_2$, + $MgCl_2$	100 %	100 %	26 %	25 %
- $(NH_4)_2SO_4$	75 %	73 %	32 %	27 %
ratio				
$MnCl_2$/ $MgCl_2$	2.1	2.0	3.8	3.9
+ $(NH_4)_2SO_4$/ - $(NH_4)_2SO_4$	1.3	1.4	3.1	3.8
+ 0.2 µg/ml α-amanitin	100 %	100 %	16 %	14 %

The complete system contains 10 mM $MgCl_2$ - 60 mM $(NH_4)_2SO_4$ - 0.02 µg α-amanitin for RNPase A and 2.5 mM $MnCl_2$ - 100 mM $(NH_4)_2SO_4$ for RNPase B in a final volume of 100 µl (30 min at 37°).

Table 3: Properties of DNA-dependent RNA polymerases in isolated nuclei from human peripheral blood leukocytes. For incubation conditions cf. legend to Tabl. 2.

alpha-amanitin and cytostatic substances like distamycin A, actinomycin D, and daunomycin. The specific activities of the DNA-directed nucleic acid polymerases are, however, significantly elevated in CLL, CML, and Hodgkin's disease stage IV, as compared to normal controls (Tab. 4). During remission, near normal values have been observed in chronic myelocytic leukemia. No elevations of the specific activities were found in reactive granulocytosis or lymphocytosis.

This phenomenon cannot be explained by a different degree of depression of endogenous DNA template. It must be assumed, that there are more polymerase molecules present in leukemic cells, or that the RNA polymerases of leukemic cells are more active than normal enzymes.

There appears to be a correlation between nucleic acid polymerase activities and the response to a cytostatic regimen. In a patient with a blastic crisis in CML, 3 mg of vincristin were applied initially. Polymerase activities fell to nearly normal values, even before peripheral leukocyte counts dropped in response to therapy. In another

patient with AML who did not respond to the COAP regimen, nucleic acid polymerase activities kept on rising, the patient became resistent to cytostatics, and died a few weeks later.

In order to verify the results obtained in the nuclear system and to further investigate normal and pathological RNA polymerase molecules, we have started to purify these enzymes. Polymerases are solubilized from isolated leukocyte nuclei by sonication and a buffer of high ionic strength (0.20 M AmS). RNA polymerase A is eluted from DEAE cellulose at 0.13 M AmS, RNA polymerase B at 0.35 M AmS. RNPase B can be further fractionated into polymerases B I and B II by re-chromatography on DEAE cellulose. B I is eluted at 0.2 M, B II at 0.35 M AmS. Using this method, RNA polymerase A has been purified 16 fold, with a specific activity of 40.3 U/mg protein, RNPase B 200 fold with a specific activity of 200 U/mg protein (1 unit = 1 pmole of labelled nucleotide incorporated/30 min 37°, Tab. 5). Parameters of the purified enzymes have been found identical to those determined in the nuclear system (Tab. 6).

A second series of experiments has been carried out in order to investigate the possibility of deranged control mechanisms in leukemic cells on the translational level. tRNA, the smallest known RNA species, exhibits a most complex structure. Numerous modifications of its bases are known which determine structure and function.

Diagnosis	RNPASE A	RNPASE B	cbRNPASE
normal leukocytes	100 % n = 9	100 % n = 9	100 % n = 8
chronic lymphatic leukemia	265 % n = 9	174 % n = 6	505 % n = 5
chronic myelocytic leukemia (untreated)	450 % n = 4	774 % n = 4	663 % n = 4
chronic myelocytic leukemia (remission)	245 % n = 4	333 % n = 4	350 % n = 4
HODGKIN `s disease (stage IV a)	215 % n = 9	288 % n = 9	220 % n = 8
FRIEND murine virus leukemia	611 % n = 4	62 % n = 4	220 % n = 4

Table 4: Specific activities of DNA-dependent RNA polymerases in nuclei from normal leukocytes and from leukocytes in malignant hematological disease. Specific activity = pmoles ^{3}H-UMP incorporated/mg protein. 45 min at 37 °C.

Table 5: Purification of DNA-dependent RNA polymerase from Friend virus murine leukemia cells.

Fraction	Total Protein	RNPase A				RNPase B			
		Total Units	Specific Activity	Yield	Purification	Total Units	Specific Activity	Yield	Purification
	mg		units / mg protein	%			units / mg protein	%	
I Homogenate	120	310	2.59	100	1	104	0.87	100	1
II S 30	48.6	245	5.04	79.0	1.95	121	2.50	116	2.87
III AmS precipitate	21.2	181	8.56	58.4	3.31	71	3.37	68.2	3.87
IV DEAE cellulose									
0.13 M eluate (A)	8.2	263	40.3	84.8	16.1				
0.35 M eluate (B)	3.1					406	204	390	236
V DEAE cellulose									
0.2 M eluate (B I)	0.24					16.4	68.4	15.8	78.6
0.35 M eluate (B II)	0.60					34.3	58.3	32.9	67.0

AmS = ammonium sulphate
1 unit = 1 pmole of labelled UMP incorporated in 30 min at 37°
Specific activity = most active fractions of column eluate.

Table 6: Properties of purified DNA-dependent RNA polymerases from human leukocytes and from Friend virus murine leukemia cells. For incubation conditions cf. legend to Tabl. 2.

RNA polymerase	A	A	B	B	B I	B II
Diagnosis	FR	FR	CML	FR	FR	FR
Conditions:	%	%	%	%	%	%
complete	100	100	100	100	100	100
- $MgCl_2$, + $MnCl_2$	121	364	100	100	100	100
- $MnCl_2$, + $MgCl_2$	100	100		18.6	75.6	54.9
ratio Mn^{2+} / Mg^{2+}	1.21	3.64		5.38	1.32	1.82
+ 0.2 ug / ml α-amanitin	100	100	2.0	3.9	2.0	2.0

The complete system contained 10 mM $MgCl_2$ - 60 mM $(NH_4)_2SO_4$ - 100 µg/ml native calf thymus DNA for RNA polymerase A and 2.5 mM $MnCl_2$ - 100 mM $(NH_4)_2SO_4$ - 100 µg/ml denatured calf thymus DNA for RNA polymerase B (30 min 37°).

Name	Diagnosis	Leuko / mm^3	Therapy	pmoles CH_3- bound / 20µg Protein		
				Lympho	Granulo	Leuko
N.N.	normal (n = 4)	6600 - 9200	-	-	-	<0,5 - 1,4
N.N.	normal (n = 13)	5000 - 7100	-	<0,5 - 3,2	< 0,5	-
N.N.	virus infection (n = 4)	7550 - 16250	antibiotics	1,4 - 2,0	< 0,5	-
F.M., 63	CML	93500	-	< 0,5	< 0,5	-
G.S., 59	"	85000	MYL	1,4	< 0,5	-
G.D., 40	"	24800	MYL	-	-	< 0,5
K.S., 46	" blast crisis	68200	MYL,URB,VCR	-	-	21,7
I.N., 49	" "	41000	MYL	-	-	6,6
I.B., 18	" "	17900	ARA-C	-	-	5,5
"	" "	2150	" + SH-G	< 0,5	< 0,5	-
P.P., 30	AML	13750	-	< 0,5	< 0,5	-
E.H., 42	"	3800	-	1,6	< 0,5	-
A.Z., 14	"	17500	-	-	-	< 0,5
"	"	5400	SH-G,URB	< 0,5	< 0,5	-
A.B., 40	"	5300	VAMP	-	-	< 0,5
A.B., 11	"	102500	SH-G,MTX,URB	-	-	1,3
"	"	53800	" " "	-	-	3,5
"	"	214000	+ ADR	-	-	< 0,5
G.V., 8	"	45500	-	-	-	6,2
"	"	57500	VCR,SH-G,URB	-	-	4,8
"	"	152500	" " "	-	-	5,2
A.K., 68	CLL	10900	URB, ENX	-	-	0,6
W.S. 65	"	46100	URB	2,8	< 0,5	-
H.M. 69	"	95500	URB	< 0,5	< 0,5	-
A.R., 7	ALL	85000	ENX	-	-	6,0
A.A., 9	"	BM	6MP, MTX	-	-	12,3
F.S., 40	"	4600	ENX	6,9	< 0,5	-
"	"	2700	"	4,2	< 0,5	-
M.G., 32	AL remission	13200	URB	1,7	< 0,5	-
N.G., 21	" "	14400	URB	< 0,5	< 0,5	-
H.P., 14	" (aleukemic)	6050	URB	-	-	1,3
J.K., 5	"	286000	URB,ADR,ARA-C	-	-	8,9

differentiation

Table 7: Specific activities of tRNA methylases in normal and pathological leukocytes.

The reaction mixture contained, in a final volume of 100 µl : 5 µmoles of Tris-HCl, pH 7.5, 1 µmole of $MgCl_2$, 0.5 µmole of dithiothreitol, 1nmoles of (^{14}C)-S-adenosyl-L-methionine (55 Ci/mole, Amersham), 2nmoles of E. coli tRNA, 5 to 50 µg of protein in the enzyme fraction. After 90 min incubation at 37 °C the tRNA was precipitated with 0,5 % cetyltrimethylammonium bromide and a second time with 2 volumes of ethanol. The (^{14}C)-methyltransfer was measured by counting aliquots of the redissolved tRNA in a Packard Tricarb liquid scintillation counter at an efficiency of 74 %. Enzyme concentration curves were determined in each case and the values of the specific activities (expressed as pmoles of CH_3-groups bound by 20 µg of protein in 90 min) were taken from the initial linear range for these curves. For the cytological and cytochemical differentiation of leukemias see Beckmann, H. and R. Neth, this symposion. A = acute, C = chronic, L = lymphatic leukemia, M = myelocytic, AL = acute undifferentiated leukemia, BM = bone marrow;

ADR = Adriamycin, ARA-C = Cytosin-Arabinosid, ENX = Endoxan, 6MP = 6-Mercapto-Purin, MTX = Methotrexat, SH-G = Thioguanin, URB = Urbason, VCR = Vincristin.

Methylation of bases is the most frequent of these modifications. Transfer ribonucleic acid methyltransferases (tRNA methylases) are strictly species specific enzymes. They transfer methyl groups from S-adenosyl-L-methionin (SAM) into definite positions of specific bases of unmethylated tRNA precursor molecules. Results regarding the biological function of tRNA methylation, indicate changes in the chargeability with amino acids, binding to ribosomes, and codon recognition of the tRNA species. Elevated activities of tRNA methylases have been found in embryonic tissues, in proliferating tissue cultures, and in malignant growth. Thus tRNA methylases seem to possess important regulatory properties during growth, differentiation and malignant transformation of eucaryotic cells (12, 13). GALLO and coworkers reported elevated tRNA methylase activities not only in leukemic but also in proliferating normal human blood cells (14). High tRNA methylase levels, thus

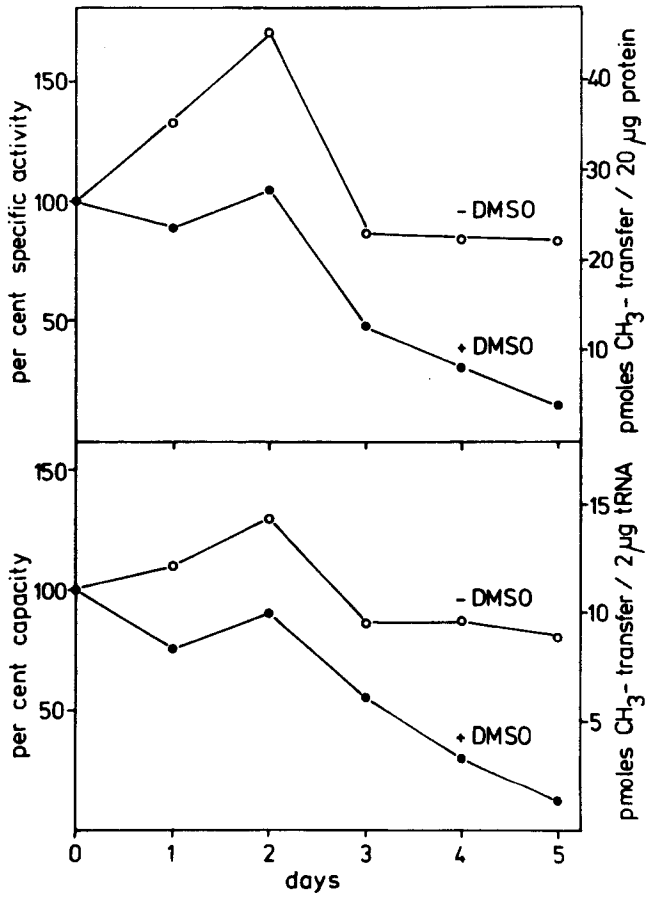

Fig. 2: Specific activity and capacity of tRNA methylases in untreated and DMSO stimulated cultures of Friend virus mouse leukemia cells.

Cells (FSD-1/clone F 4) were grown in Eagle's medium with Earle's balanced salt solution, supplemented with twice the usual amount of amino acids and vitamins, five times the usual amount of glutamine and 15 % foetal calf serum. Cultures were started with 200 cells/µl. On day 0 DMSO was added to a final concentration of 1.5 % and the culture flasks were closed. Controls without DMSO were treated in the same way. Cells were harvested by centrifugation, disrupted by sonication and tRNA methylases were measured as described in the legend to table 7 except that additional capacity experiments were performed with limiting amounts (0.04 nmoles) of tRNA. Values are expressed in per cent activity of that at day 0 (= 100 %).

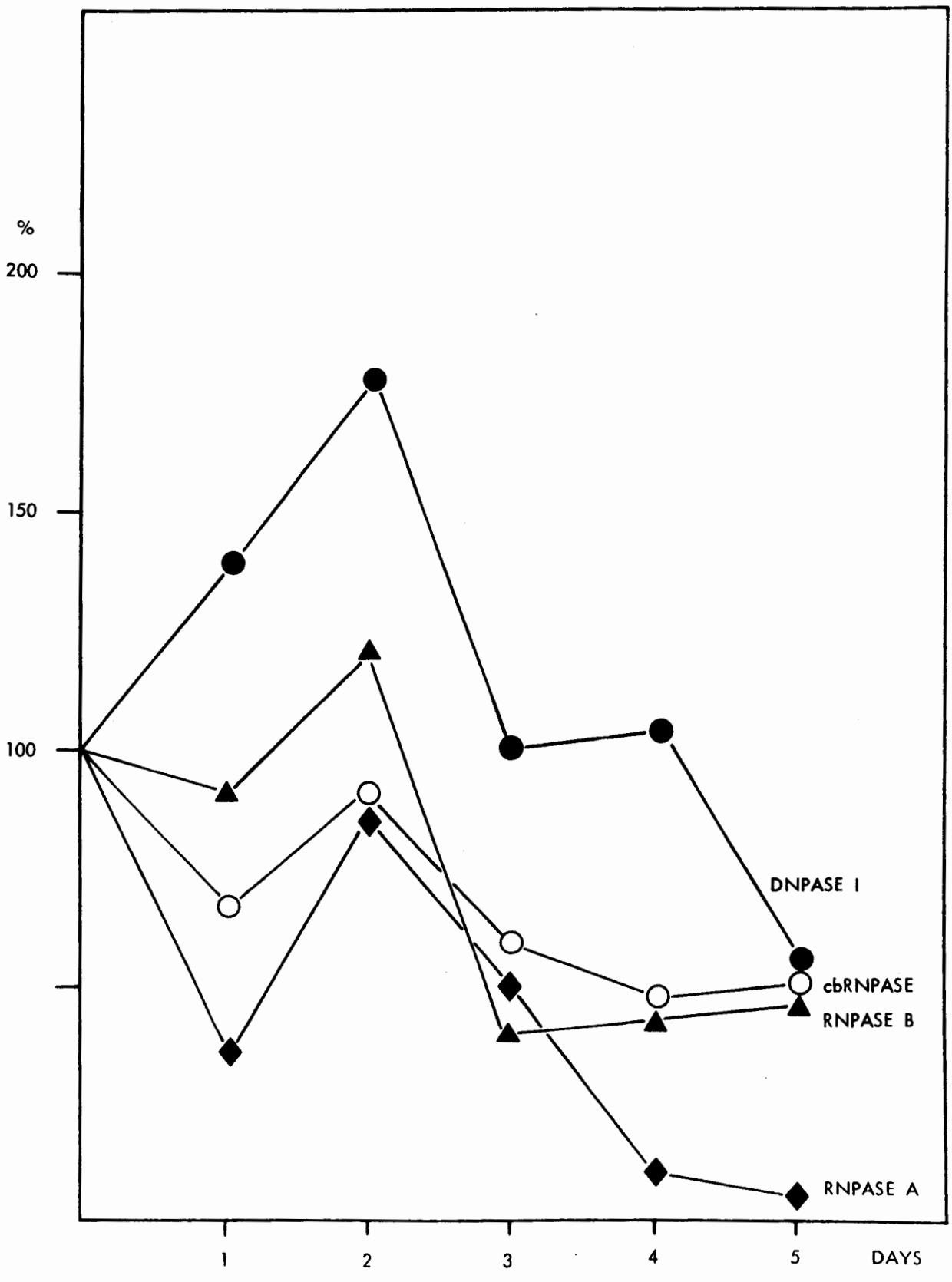

Fig. 3: DMSO-stimulated Friend virus murine leukemia cells: polymerase activities in percent of control cultures. For culture conditions cf. legend to Fig. 2.

appear to be an indicator of proliferation in general rather than of malignancy per se. Tab. 7 shows the specific activities of tRNA methylases in normal and pathological white blood cells (for details of methods cf. 10). Normal, unfractionated leukocytes exhibit very low methylase activities, sometimes even below the sensitivity of the assay. Normal lymphocytes, however, always show measurable activities. In virus induced lymphocytosis, there were no increases in lymphocyte tRNA methylase activities as compared to normal controls. We never observed demonstrable methylase activities in isolated granulocytes in all patients investigated so far. In CML, tRNA methylases have been found elevated in blastic crisis, in AML in the most undifferentiated forms only. tRNA methylase activities are elevated in some cases of CLL, and always in ALL. Of the four cases of acute undifferentiated leukemia (AL), investigated as yet, two cases in remission and one newly detected aleukemic case exhibited low activities. A fourth case, examined in the final stage of the disease, was strongly positive. No correlation has been observed between the number of white blood cells in the peripheral blood and tRNA methylase activities. Regarding high tRNA methylase activities as a sign of rapid proliferation, i. e. immaturity, a decrease of methylase activities should be expected, when immature leukemic cells further differentiate, spontaneously or after exogenous stimulation. A suitable in vitro model for investigation of this hypothesis are the DMSO-stimulated Friend virus leukemia cells. These cells start to synthesize hemoglobin three days after addition of 1.5 % DMSO (15). Hemoglobin production reaches its maximum after five days. Fig. 2 shows that there is a decrease of the specific activities as well as of the capacities of tRNA methylases in DMSO-stimulated cells after day three, when the exponential growth phase is terminated. At the same time, control cultures exhibit persisting levels of enzyme activities. Simultaneous determination of DNA-dependent DNA and RNA polymerase activities in these cells (Fig. 3) demonstrates an immediate increase of DNA polymerase I (for details of methods cf. 16) after addition of DMSO, reaching a maximum on the second day. RNA polymerase B reaches its maximum activity also on the second day with about 120 % of the activity of the control cultures. The enzyme responsible for rRNA and tRNA synthesis, RNA polymerase A, never exeeds the level of the control cultures. From day three to five, while increasing numbers of hemoglobin positive cells are appearing, DNA polymerase I, RNA polymerase B, and the chromatin bound RNA polymerase activities approximate 50 % of control values, while RNA polymerase A drops to nearly zero, as well as the tRNA methylase activities.

In conclusion, elevated tRNA methylase activities, determined in native leukocytes, do not indicate the malignancy of hematological disease in general, but appear to be found when differentiation of leukemic cell lines is blocked at an early, undifferentiated stage.

The results about DNA dependent RNA polymerases in human leukocytes suggest a correlation of enzyme activities and the clinical course of the disease and the response to therapy. They have been found elevated in leukocytes in all malignant hematological diseases investigated so far, decreasing during successful cytostatic therapy and in remission.

We hope, that further analysis of biochemical parameters in the course of human leukemias will contribute new parameters for diagnosis, therapy, and prognosis in addition to the classical morphological and cytochemical criteria.

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