STUDIES ON PURINE METABOLISM IN RELATION TO LEUKEMIA AND LYMPHOID CELL DIFFERENTIATION

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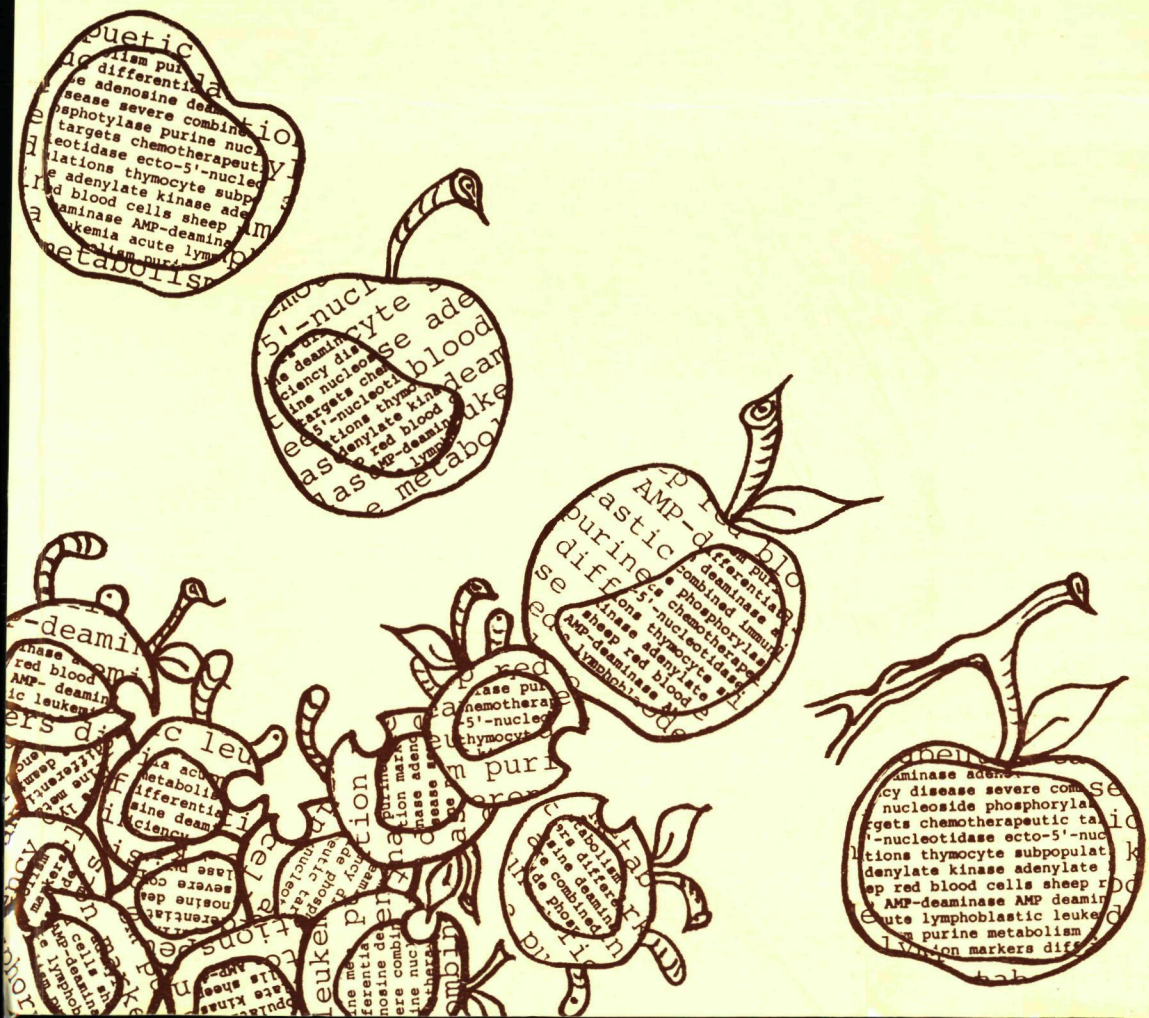

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Aan mijn ouders

Voor Corine

ABBREVIATIONS

ADA	- adenosine deaminase
AdKin	- adenylate kinase
ADP	- adenosine diphosphate
AK	- adenosine kinase
ALL	- acute lymphoblastic leukemia
AMP	- adenosine monophosphate
AMPD	- AMP deaminase
ANAE	- α-naphtyl acetate esterase
AOPCP	- α,β-methyleneadenosine-5'-diphosphate
ara-C	- cytosine arabinoside
ATP	- adenosine triphosphate
B-ALL	- ALL with B cell phenotype
B-CLL	- CLL with B cell phenotype
BSA	- bovine serum albumin
cALL	- ALL with the "common" phenotype
cAMP	- cyclic AMP
CDP	- cytidine diphosphate
cIgM	- cytoplasmic IgM heavy chains
CLL	- chronic lymphocytic leukemia
CMP	- cytidine monophosphate
ConA	- concanavalin A
CTP	- cytidine triphosphate
dCF	- deoxycoformycin
dCK	- deoxycytidine kinase
DNA	- deoxyribonucleic acid
EDTA	- ethylene diamino tetraethyl acetate
EHNA	- erythro-9-(2-hydroxy-3-nonyl)adenine
E-rosettes	- rosettes with sheep red blood cells
FAB	- French American British
FCS	- foetal calf serum
FITC	- fluorescein isothiocyanate
5FdUMP	- 5-fluoro-deoxyUMP
5FU	- 5-fluorouracil
GDP	- guanosine diphosphate
GMP	- guanosine monophosphate
GTP	- guanosine triphosphate
Hepes	- N-2-hydroxyethylpiperazine-N'-2 ethane sulfonic acid
HGPRT	- hypoxanthine-guanine phosphoribosyltransferase
IgA	- immunoglobulin A
IgG	- immunoglobulin G
IgM	- immunoglobulin M
IMP	- inosine monophosphate
LUC	- large unstained cells
MEM	- minimal essential medium
6MP	- 6-mercaptopurine
MTX	- methotrexate
nonBnonT-ALL	- ALL with neither the B cell nor the T cell phenotype
5'NT	- purine-5'nucleotidase
OMP	- orotidine monophosphate
OPRT	- orotate phosphoribosyltransferase
PBL	- peripheral blood lymphocytes
PEI	- polyethyleneimine
PHA	- phytohaemagglutinin

Pi	- phosphate ion
Pipes	- piperazine-N,N'-bis(2-ethane sulfonic acid)
PMC	- parafilm microcuvette
POPOP	- 2,2'-p-phenylene-bis(5-phenyloxazole)
PPO	- 2,5-dephenyloxazole
PreB-ALL	- ALL with the preB phenotype
PRPP	- phosphoribosyl pyrophosphate
PWM	- pokeweed mitogen
R-5-P	- ribose-5-phosphate
RPMI	- Roswell Park Memorial Institute
SCID	- severe combined immunodeficiency disease
s.d.	- standard deviation
sIg	- surface immunoglobulin
SLE	- systemic lupus erythematosus
SpA	- protein A from Staphylococcus aureus
spec. act.	- specific activity
T-ALL	- ALL with the T cell phenotype
TDP	- thymidine diphosphate
6TG	- 6-thioguanine
TMP	- thymidine monophosphate
tris	- tris(hydroxymethyl) aminomethane
TRITC	- tetramethyl rhodamine isothiocyanate
TTP	- thymidine triphosphate
T_γ	- T cells with a receptor for the Fc portion of IgG
T_μ	- T cells with a receptor for the Fc portion of IgM
$T\text{-}T_\gamma$	- T cells depleted from T_γ cells
UDP	- uridine diphosphate
UMP	- uridine monophosphate
unc.-ALL	- unclassified-ALL
UTP	- uridine triphosphate
XLA	- X-linked agammaglobulinaemia
XO	- xanthine oxidase

chapter 1

INTRODUCTION

The association of genetically determined disturbances of purine metabolism with defects of the immune system is well documented(10,11, 23). Inherited deficiencies of adenosine deaminase(ADA), purine nucleoside phosphorylase(PNP) and purine-5'nucleotidase(5'NT) are related to dysfunction of different lymphoid subpopulations. ADA deficiency is associated with a combined T and B cell defect(10); very low PNP activities were found in patients with a T cell dysfunction(11), whereas lowered 5'NT activities were observed in patients with X-linked agammaglobulinaemia(7). Recently PNP activity was reported to be decreased in patients with systemic lupus erythematosus(18), a disease in which decreased numbers of T suppressor cells have been encountered(15). Although several hypotheses can be brought forward, the exact pathophysiological mechanism(s) leading to the immune defects are still not completely understood (21). Studying purine metabolism in isolated lymphoid subpopulations might not only contribute to our knowledge of pathophysiological mechanisms in immunodiseases, but also to a better understanding of the role of purine metabolism in normal lymphoid cell function.

Lymphoblastic leukemia is considered to be a malignant disorder of lymphocyte differentiation, the malignant transformation occurring at various stages of normal lymphoid differentiation(13,14). The latter leads to the different immunological subtypes of acute lymphoblastic leukemia(13). However, cells from normal differentiation stages(e.g. stem cells, committed progenitor cells, pre-thymocytes, etc.) are not or hardly available for investigation. Studying purine metabolism in the malignant counterpart of these normal differentiation stages might give more information about the relation of purine metabolism and normal lymphoid differentiation. Moreover, it might become clear why in some immunodeficiency diseases lymphoid cells from a certain lineage or differentiation stage are affected. The outcome of these studies may also provide further diagnostic tools, especially for the discrimination of subclasses of leukemias.

In anti-leukemia chemotherapy, many of the drugs used have a rather wide spectrum of action(19); they are not only affecting the leukemic cells but also apparently healthy other tissues. Some of these anti-leukemic drugs are purine or pyrimidine derivatives or are interfering with purine- and pyrimidine metabolism(8).

Wellknown purine and pyrimidine related anti-leukemic drugs are 6-mercaptopurine(6MP), 6-thioguanine(6TG), methotrexate(MTX) and cytosine-arabinoside(ara-C). Although a certain knowledge exists regarding the mode of action of these compounds(4,6,20,27), in fact little is known about the tissue specificity of this action. Part of the present study was therefore undertaken in order to explore possibilities for anti-leukemic drugs with a better, higher degree of specificity.

ENZYMOLOGICAL ANALYSIS OF PURINE METABOLISM IN LYMPHOID CELLS

In this section the development of a micromethod and the application of this method to assay enzyme activities in different lymphoid subpopulations is described. The development of such a micromethod was necessary because systematically assaying purine interconversion enzymes with conventional methods would require too many cells. The amount of peripheral blood available from volunteers and the small recovery of cells derived when fractionating peripheral blood lymphocytes or other lymphoid tissues would become prohibitive. Moreover, sometimes it was not possible to obtain large quantities of leukemic cells from patients with childhood acute lymphoblastic leukemia(ALL). Some of these patients appeared to be leukopenic(cf. chapter 7).

Conventional analysis of purine enzyme activities requires 10^5 to 10^8 cells per assay(5,7). The described micromethod, using 1,000 to 6,000 cells per assay, enabled us not only to systematically analyse purine enzyme activities in childhood ALL, but also in different subfractions of human lymphoid cells(chapters 4,5,6,7 and 9). The effect of different methods of cell destruction on the activity of purine enzymes was tested(chapter 2). The best results were obtained with lyophilization of the cells and subsequent resuspension in a buffer containing the detergent triton X-100. This method of cell destruction was used throughout the studies outlined in this thesis. The effect of the addition of bovine serum albumin to the cell suspensions on the counting efficiency and on the enzyme activities measured was studied (chapter 3).

Our micromethod was employed in studies on different lymphoid cell subpopulations(chapters 4 and 5). The differences in purine enzyme activities between T and B cells and between T_γ and T-T_γ cells were assessed. The effect of differences in isolation procedures of lymphoid cells on purine enzyme activities are discussed mainly in chapter 5. One of the major conclusions from this set of experiments was, that the presence or absence of bloodplatelets in the lymphoid cell preparations could cause considerable differences in enzyme activities. The lymphoid cell preparations used in all other experiments described in this thesis were essentially platelet-free.

EXPRESSION OF PURINE METABOLISM IN LEUKEMIA

The study of purine metabolism in lymphoblastic leukemia might serve three purposes:

1. The search for additional diagnostic biochemical markers in lymphoblastic leukemia.
2. The search for a relation between biochemical markers of lymphocytic differentiation and the immunological markers.
3. Based on the specific deviant enzymatic make-up of the malignant lymphoblasts, possibilities for specific chemotherapy might be explored.

Chapter 6 contains a brief outline of differences in purine enzyme activities found in two major subclasses of ALL. Namely the T-ALL subclass, mainly characterized by the receptor for sheep red blood cells (E-rosettes), and the nonBnonT-ALL subclass, which has neither immunoglobulins on the cell membrane(nonB) nor the E-rosette marker(nonT). From a clinical point of view this major subdivision has a prognostic value: nonBnonT-ALL patients might have a better prognosis than T-ALL patients(1,3). We were able to study these patients in sufficient numbers and to quantify the differences in purine enzyme activities.

A number of additional markers are available at present. Greaves et al.(13) described four phenotypic subgroups of ALL. T-ALL is phenotypically defined by the presence of the E-rosette marker, expression of T cell antigen and a relatively high activity of terminal

deoxynucleotidyltransferase and acid phosphatase. Lymphoblasts of B-ALL patients have immunoglobulins on their cell membrane. The group of ALL patients classified earlier as nonBnonT-ALL patients could be subdivided on the basis of the presence of a common ALL(cALL) antigen. Greaves et al.(2,12) have developed an antiserum against this antigen, which is widely used now. NonBnonT-ALL patients in which the cells react positively with this antiserum are designated as cALL patients. The nonBnonT-ALL patients that could not be classified neither with the E-rosette marker nor with the presence of immunoglobulins on the cell membrane, nor with the cALL marker are designated unclassified(unc.-ALL). Vogler et al.(25)described a number of cALL patients, who had cytoplasmic IgM heavy chains present in their leukemic cell population. Since this marker appears to be related to preB cells(9), these cALL patients were classified as preB-ALL. Recently an ALL subclass was described with a mixed common/T phenotype(17).

In order to serve the three purposes mentioned above we have studied enzyme activities of purine metabolism in relation with the E-rosette marker, the cALL marker, the presence of immunoglobulins on the cells and the preB cell marker in patients with acute lymphoblastic leukemia(chapter 7). Since the group of B-CLL patients is likely to represent another stage of normal lymphoid differentiation(13) we also studied a group of B-CLL patients(chapter 8).

NUCLEOSIDE TOXICITY IN MATURE AND IMMATURE LYMPHOID CELLS

The differences in enzyme activities observed in different groups and subgroups of lymphoblastic leukemia appeared to be considerable. Differences in the activity levels of ADA, PNP, 5'NT and adenylate kinase(AdKin) appeared to be most pronounced(chapter 6,7,8). Since the handling of purines in the leukemic subgroups was so different, we studied intoxication of cells with an enzymatic make-up comparable with a certain subgroup of leukemia for intoxication with different nucleosides. As thymocytes may be considered as normal equivalents of certain T-ALL cells, we performed a set of toxicity studies with thymocytes. The toxicity was related to enzyme activities. From our results it was clear that some purine enzymes could probably serve as markers for

different subgroups of ALL. In addition we studied the possibilities of using purine enzyme activities as markers for differentiation in the intrathymic situation(chapter 10).

Differences in nucleoside toxicity were also studied using mature peripheral blood lymphocytes, stimulated with mitogens known to stimulate different lymphoid subpopulations(16). The effects of the nucleosides adenosine and deoxyadenosine on DNA synthesis, protein biosynthesis and IgG secretion were studied. Although the exact mechanisms are not yet clear and the limitations of working with stimulated lymphocytes are known to us, it was indicated that different subpopulations of mature peripheral blood lymphocytes, including T_{μ} and T_{γ}, have a different sensitivity towards the nucleosides mentioned. Moreover, depending on the concentrations used, deoxyadenosine exerts a biphasic effect on poke weed mitogen stimulated peripheral blood lymphocytes(chapter 10).

REVIEW

Finally, in the last chapter, an overview is given of the evidence available to date regarding the relation between purine metabolism, leukemia and lymphoid cell differentiation. Reports from literature are reviewed and considered in relation to the findings reported in this thesis.

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chapter 2

MICROMETHODS FOR THE MEASUREMENT OF PURINE ENZYMES IN LYMPHOCYTES

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ABSTRACT

A new micromethod enabling the characterization of several enzymes of purine metabolism in small numbers(500-5,000) of lymphocytes is presented. This method is based on ultramicrotechniques, making use of radioactive substrates, which have been publsihed previously.

The isolation of lymphocytes from peripheral blood by nylon wool filtration and Ficoll-Isopaque density centrifugation yielded a lymphocyte fraction of about 98% purity. The lymphocyte suspensions were diluted to 10^5 to 10^6 cells/ml and aliquots of $\frac{1}{2}$ µl were pipetted into small incubation vessels, moulded in parafilm immediately before use (Parafilm-Micro-Cuvette; PMC). The cells were frozen and lyophilized overnight. This procedure yielded maximum enzyme activities as compared to sonification and freezing-thawing. Incubations were routinely done in fivefold; they were started by addition of 3 µl of the appropriate incubation mixture to each PMC. After incubation, the contents of the PMC were pressed directly on chromatography paper. Products and substrates were separated by descending chromatography or high voltage electrophoresis.

The present micromethod allows to assay reproducibly with less than 100,000 cells(500-5,000 cell/incubation) six purine interconversion enzymes, including hypoxanthine-guanine phosphoribosyltransferase, adenine phosphoribosyltransferase, adenosine deaminase, purine nucleoside phosphorylase, adenosine kinase and purine-5'nucleotidase.

INTRODUCTION

The involvement of purine interconversion enzyme defects in impairment of the immune system is now well documented(5,6,7). Although it has been suggested that deoxypurine nucleotides might be the toxic metabolites in these immune diseases(1,2), the mechanism which leads to dysfunctions of T or B cells, or both of them, is still not completely elucidated. A better understanding of purine interconversions in B and T cell subfractions might help to obtain a better view on B or T cell specificity in these immune diseases. One of the possibilities to achieve this might be a systematic analysis of purine metabolism in T and non-T lymphocytes.

The first purpose of the present study was to investigate the effect of cell destruction procedures, such as sonification and lyophilization, on a number of purine enzyme activities. This was studied with pure lymphocyte preparations from human tonsils.

Determination of nine purine enzyme activities in lymphocyte subfractions from one peripheral blood sample using conventional methods required too much blood. Therefore, the second purpose of the present study was to develop new radiochemical micro techniques, which are based on previously described ultramicrochemical methods(3,8,9,10,14,15). This method has been applied to almost pure, unfractionated lymphocyte preparations, using 500 to 5,000 cells per assay.

MATERIALS AND METHODS

Isolation of Lymphocytes from Tonsils

After removal of the surrounding tissues, tonsils were homogenized (Potter; Janke & Kunkel KG) in tris-buffered minimal essential medium (MEM; Gibco, F-14; pH 7.4) containing 15%(v/v) foetal calf serum(FCS; Gibco). After filtration over a wire mesh, the suspension was layered on Lymphoprep(gravidity 1.077 gr/ml; Nyegaard AS, Oslo). Lymphocytes were collected from the interphase after centrifugation for 20 min. at 1,000 g(room temperature) and washed once with a solution containing

155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM disodium EDTA(pH 7.4). Before use the cells were washed twice with 0.9% NaCl(w/v).

Isolation of Lymphocytes from Peripheral Blood

Thirty ml of defibrinated(on glass beads; Ø 5 mm) venous blood was incubated for 15 min. at 37^0 C. After passage through a nylon wool column(4) the effluent was diluted with MEM/tris containing 15% FCS(v/v) to a concentration of approximately 10^9 blood cells/ml. Subsequently this cell suspension was carefully layered on top of a density gradient, that consisted of 15 ml Ficoll-Isopaque(gravidity 1.085 gr/ml; Ficoll 400, Pharmacia, Uppsala, Sweden; Isopaque 440 mg J/ml, Nyegaard & Co. AS, Oslo) and 5 ml of a lighter Ficoll-Isopaque solution(gravidity 1.055 gr/ml). The centrifugation was carried out at room temperature(20 min., 1,000 g). Lymphocytes could be collected from the interphase between the two Ficoll-Isopaque solutions. During the isolation the amounts of monocytes, granulocytes and lymphocytes were checked with a Hemalog D (Technicon Instruments Corp., Tarrytown, NY, USA). The original total leukocyte fraction in whole EDTA blood contained 29% lymphocytes, 7% monocytes, 62% granulocytes and 6% "large unstained cells"(LUC). Defibrination and filtration over a nylon wool column according to de Pauw et al.(4) yielded a fraction after Ficoll-Isopaque density centrifugation which contained over 97% lymphocytes and a very low contamination of monocytes, granulocytes and LUC.

"Macro" Enzyme Assays with Tonsillar Lymphocytes

Lymphocytes were lysed in several ways: freezing and thawing(-20^0 C, 5 cycles), lyophilization and sonification(3 x 10 sec., output control 7; Sonifier B-12, Branson Sonic Power Co., Danbury, CT, USA). For all enzyme assays 0.05 to 5 µgr protein per incubation was added(protein estimation according to Lowry et al., 12). All enzyme assays were carried out in triplicate and in table 1 the mean values are given. Hypoxanthine-guanine phosphoribosyltransferase(HGPRT; E.C. 2.4.2.8) and adenine phosphoribosyltransferase(APRT; E.C. 2.4.2.7) were assayed essentially according to De Bruyn et al.(3). Purine nucleoside phosphorylase(PNP;

E.C. 2.4.2.1) and adenosine deaminase(ADA; E.C. 3.5.4.4) were assayed essentially according to previously described methods(14,15).

"Micro" Assays with Peripheral blood Lymphocytes

Lymphocyte suspensions containing 1,000 to 10,000 cells/µl were prepared in 0.9% NaCl. Aliquots of 0.5 µl were pipetted into small incubation vessels prepared from parafilm(Parafilm "M", American Can Co., Greenwich, CT, USA). These parafilm micro cuvettes(PMC) were prepared immediately before use(8,9,10). The lymphocytes were frozen in the PMC's at -20^0 C for 15 to 30 min. and subsequently lyophilized overnight. All enzyme assays were carried out in five-fold. For HGPRT, APRT, ADA and PNP the reactions were started by adding 3 µl of the appropriate incubation mixture. The concentrations were the same as described above for the "macro" assays except for the addition of 0.2%(v/v) triton X-100 (Sigma) in several experiments. In the HGPRT reaction also 8-^{14}C-guanine(0.13 mM; specific activity 55 mCi/mmol; Radiochemical Centre Amersham, UK) was used as a substrate; ADA was also tested with 8-^{14}C-deoxyadenosine(0.26 mM; specific activity 45 mCi/mmol; NEN chemicals GmbH, Dreieich, FRG). Incubation times were 1 to 4 hours at 37^0 C. Quantification of enzyme activities was carried out as described elsewhere(3,14,15). The adenosine kinase(AK; E.C. 2.7.1.20) assay was adapted from Meyskens and Williams(13). To the lyophilized lymphocytes, 3 µl of a reaction mixture was added, containing 3 µM 8-^{14}C-adenosine, 1.5 mM ATP(Boehringer Mannheim), 0.3 M trisodiumacetate/acetic acid(pH 5.7), 0.6 mM $MgCl_2$, 12.5 µM erythro-9-(2-hydroxy-3-nonyl)-adenine(EHNA), kindly supplied by Dr. H.A. Simmonds(Purine Laboratory, Guy's Hospital Medical School, London, UK) and 0.2% triton X-100. After incubation(4 hours at 37^0 C) separation of substrate and products was performed by means of high voltage electrophoresis on Whatmann 3 MM paper(0.05 M citrate buffer, pH3.9; 70 V/cm). Purine-5'nucleotidase(5'NT; E.C. 3.1.3.5) assay; to the lyophilized lymphocytes 3 µl of a reaction mixture was added(11) containing 0.05 mM tris/HCl(pH 8.5), 0.02 M $MgCl_2$, 6.25 mM 2-glycerophosphate(Sigma) and 0.6 mM 8-^{14}C-AMP(specific activity 61 mCi/mmol; Radiochemical Centre Amersham, UK). Incubation(4 hours at 37^0 C) was followed by separation of substrate and products as in the AK assay.

Table 1: Effect of Different Methods of Cell Destruction on 5 Purine Enzyme Activities

Enzyme	Procedure							
	1	2	3	4	5	6	7	8
HGPRT	4.3	5.9	4.6	6.1	2.9	5.2	4.4	4.9
APRT	7.4	8.9	7.2	9.6	7.4	8.3	8.5	7.7
ADA	49.4	85.0	52.2	84.0	54.7	69.2	51.2	81.2
PNP-Hx	44.0	90.5	71.8	110.6	55.0	33.0	51.1	57.6
PNP-Ino	15.2	21.8	23.5	26.0	20.0	15.4	20.4	24.7

Procedure 1. freezing and thawing(5 cycles, -20^0 C)
2. freezing and thawing(5 cycles, -20^0 C), with addition of 0.2% triton X-100
3. lyophilization and resuspension in 0.01 M tris/HCl(pH 7.4)
4. lyophilization and resuspension in 0.01 M tris/HCl(pH 7.4), with addition of 0.2% triton X-100
5. lyophilization and sonification after resuspension in 0.01 M tris/HCl(pH 7.4)
6. sonification preceded by lyophilization
7. sonification
8. sonification in the presence of 0.2% triton X-100

Enzyme activities are expressed in 10^{-9} moles product formed/10^6 cells. hour. For each enzyme the procedure which gives the highest activity is underlined.

RESULTS AND DISCUSSION

Effect of Various Lysate Preparations on Enzyme Activities

Tonsillar lymphocytes, suspended in 0.01 M tris/HCl(pH 7.4) were lysed in eight different ways in order to investigate, which method of cell destruction is to be preferred for the determination of purine enzyme activities(table 1). After preparation of the lysate, insoluble particles were removed by centrifugation(300 g, 15 min.). As can be seen in table 1 the procedures using 0.2% triton X-100 yielded highest enzyme activities, especially in the case of freezing and thawing and lyophilization(procedures 2 and 4). This effect was seen with all enzymes tested.

In the present studies enzyme activities are expressed on a per cell basis. When expressing the activities on a protein basis it was found that, although with some procedures much more protein was released into the soluble fraction than with other procedures(e.g. lyophilization +

Table 2: Reproducibility of the "Micro" Assay for ADA and PNP, Assayed in Five-Fold in the Same Sample

	probe no.	net product formed (cpm)	enzyme activity (10^{-9} moles/10^6 cells.hour)
ADA	1	6568	57.77
	2	5620	62.95
	3	6651	53.40
	4	4598	51.29
	5	7302	71.04
			mean ± s.d. 59.29 ± 7.95
PNP	1	6591	80.00
	2	8508	78.51
	3	8549	90.16
	4	6132	80.73
	5	7302	83.72
			mean ± s.d. 82.62 ± 4.62

ADA assay: incubation with 1,500 cells for 4 hours at 37^0 C, input 60,000 cpm 8-^{14}C-adenosine.
PNP assay: incubation with 1,500 cells for 1 hour at 37^0 C, input 30,000 cpm 8-^{14}C-hypoxanthine.

sonification; procedure 5), the specific enzyme activities were not higher as compared to other procedures. For routine determinations the method of choice for the preparation of lymphocyte lysates turned out to be lyophilization with addition of 0.2% triton X-100(procedure 4).

Table 3: Purine Interconversion Enzymes in Lymphocytes

Enzyme	Substrate	Specific Activity ± s.d.
HGPRT	hypoxanthine	2.95 ± 0.72
	guanine	6.49 ± 2.44
APRT	adenine	8.93 ± 1.56
ADA	adenosine	46.27 ± 10.94
	deoxyadenosine	34.36 ± 9.04
PNP	hypoxanthine	63.87 ± 16.70
	inosine	12.99 ± 1.82
AK	adenosine	0.74 ± 0.47
5'NT	AMP	12.26 ± 7.08

Activities calculated from a group of 7 healthy individuals and expressed as 10^{-9} moles/10^6 cells.hour.

Nine purine interconversion enzyme activities could reproducibly be assayed with relatively small numbers of cells(500 to 5,000). Two examples are given in table 2, where the raw data and calculated enzyme activities are given of an ADA and PNP assay; measurements were carried out in five-fold.

The mean activities in purified normal human lymphocytes of nine enzymatic reactions involved in purine interconversions are shown in table 3. ADA and PNP displayed the highest activity in pure lymphocytes, whereas relatively low activities were found for HGPRT and AK. The rather wide range of specific enzyme activities was attributed to individual variation rather than to methodological errors.

With the present "micro" method it becomes possible to carry out a great number of enzyme determinations with small numbers of cells. In the present study only radiochemical assays are described, but also other substrates(e.g. fluorogenic; Van Laarhoven et al. unpublished) can be employed both for experimental and diagnostic purposes.

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chapter 3

ENZYMES OF PURINE NUCLEOTIDE METABOLISM IN HUMAN LYMPHOCYTES

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ABSTRACT

A method is presented for systematic analysis of purine enzymes in small lymphocyte subfractions. For the determination of 7 different enzymes of purine metabolism(hypoxanthine-guanine phosphoribosyltransferase, HGPRT; adenine phosphoribosyltransferase, APRT; adenosine deaminase, ADA; purine nucleoside phosphorylase, PNP; adenosine kinase, AK; 5'nucleotidase, 5'NT; AMP deaminase, AMPD) less than 200,000 peripheral blood lymphocytes are needed. In micro-incubation vessels(3 µl), 1,000 to 6,000 lyophilized lymphocytes are incubated with radioactive substrates for 15 to 180 min. Separation of substrates and products is achieved by thin-layer chromatography on PEI-cellulose. Addition of BSA to the incubation mixtures results in higher specific enzyme activities and narrower ranges of mean values of a control group.

INTRODUCTION

As a consequence of adenosine deaminase(ADA) and purine nucleoside phosphorylase(PNP) deficiency, accumulation of deoxypurine nucleotides, especially deoxyATP and deoxyGTP, may lead to dysfunction of T and/or B lymphocytes(1), although the selective toxicity of deoxynucleotides towards T or B cells is still not completely understood. Purine-5'nucleotidase(5'NT) is believed to play a major role in nucleotide breakdown. Therefore this enzyme may be of crucial importance in the detoxification of deoxynucleotides. Decreased 5'NT activities have been observed in patients with congenital primary agammaglobulinaemia(4).

Differences in purine interconversion pathways in T and B lymphocytes might account for the selective toxicity of deoxypurine nucleotides. Therefore, systematic analysis of purine interconversion enzymes in these different cell types seems to be of interest.

Systematic analysis of purine metabolism in peripheral T and non-T cells by conventional methods requires at least 100 ml of blood. We present here simple micro-methods for determining enzyme activities in lymphocytes obtained from only 5 to 10 ml of blood, and permitting enzymatic analysis of small subfractions of lymphocytes. For each assay, 1,000 to 6,000 cells are used in incubation volumes of 3 µl. In addition, the effect of bovine serum albumin on these micro-assays has been investigated.

MATERIALS AND METHODS

Isolation of Lymphocytes from Peripheral Blood

Venous blood(5 to 10 ml) was defibrinated and passed over a nylon wool column as described by De Pauw et al.(11). The resulting cell suspension was diluted with 2 volumes MEM/tris containing 15%(v/v) foetal calf serum(FCS) which had been heat inactivated and screened for virus and mycoplasma. Subsequently this cell suspension was carefully layered on top of a density gradient, consisting of 15 ml Ficoll-Isopaque(gravity 1.085 gr/ml, Ficoll 400, Pharmacia, Uppsala, Sweden, Isopaque 440 mg J/ml,

Nyegaard and Co., AS, Oslo) and 5 ml of a lighter Ficoll-Isopaque solution(gravity 1.055 gr/ml). The centrifugation was carried out at room temperature(20 min., 1,000 g). Lymphocytes were collected from the interphase between the two Ficoll-Isopaque solutions. During isolation, the numbers of monocytes, granulocytes and lymphocytes were checked with a Hemalog D(Technicon Istruments Corp., Tarrytown, NY, USA).

Micro-Assays with Peripheral Blood Lymphocytes

Lymphocyte suspensions containing 2 x 10^6 to 12 x 10^6 cells/ml were prepared in 0.9%(w/v) NaCl in Eppendorf micro-test tubes(Eppendorf Geräte-bau, Hamburg, F.R.G.). Aliquots of 0.5 µl were pipetted into small incubation vessels prepared from parafilm(Parafilm "M", American Can Co., Greenwich, CT, USA). These parafilm micro-cuvettes(PMC) were prepared immediately before use(fig. 1; 5,6,7). The lymphocytes were frozen in the PMC's at -20^0 C for 15 to 30 min. and subsequently lyophilized overnight. All enzyme assays were carried out in quintuplicate. The reactions were started by adding 3 µl of the appropriate incubation mixture.

Hypoxanthine-guanine phosphoribosyltransferase(HGPRT; E.C. 2.4.2.8) and adenine phosphoribosyltransferase(APRT; E.C. 2.4.2.7) were assayed essentially according to De Bruyn et al.(2) and Van Laarhoven et al.(18). In the HGPRT reaction 8-^{14}C-hypoxanthine(0.13 mM; specific activity 55 mCi/mmol; Radiochemical Centre Amersham, RCA, UK) was used as a substrate. Purine nucleoside phosphorylase(PNP; E.C. 2.4.2.1) and adenosine deaminase(ADA; E.C. 3.5.4.4) were assayed according to previously described methods(15,16). ADA was tested with 8-^{14}C-adenosine(0,26 mM; specific activity 58 mCi/mmol; RCA) and 8-^{14}C-deoxyadenosine(0,26 mM; specific activity 45 mCi/mmol; NEN Chemicals GmbH, Dreieich, F.R.G.). Reactions were allowed to proceed for 15 to 90 min. at 37^0 C.

Quantification of enzyme activities was carried out by separating products and substrates by means of thin-layer chromatography. For HGPRT, APRT and PNP with 8-^{14}C-inosine(specific activity 60 mCi/mmol, RCA) as a substrate(PNP-Ino) and with 8-^{14}C-hypoxanthine as a substrate(PNP-Hx), PEI-cellulose plates(Macherey-Nagel; SEL 300 PEI), prewashed overnight in water, were used with 0.2 M NaCl as the solvent. Unlabelled

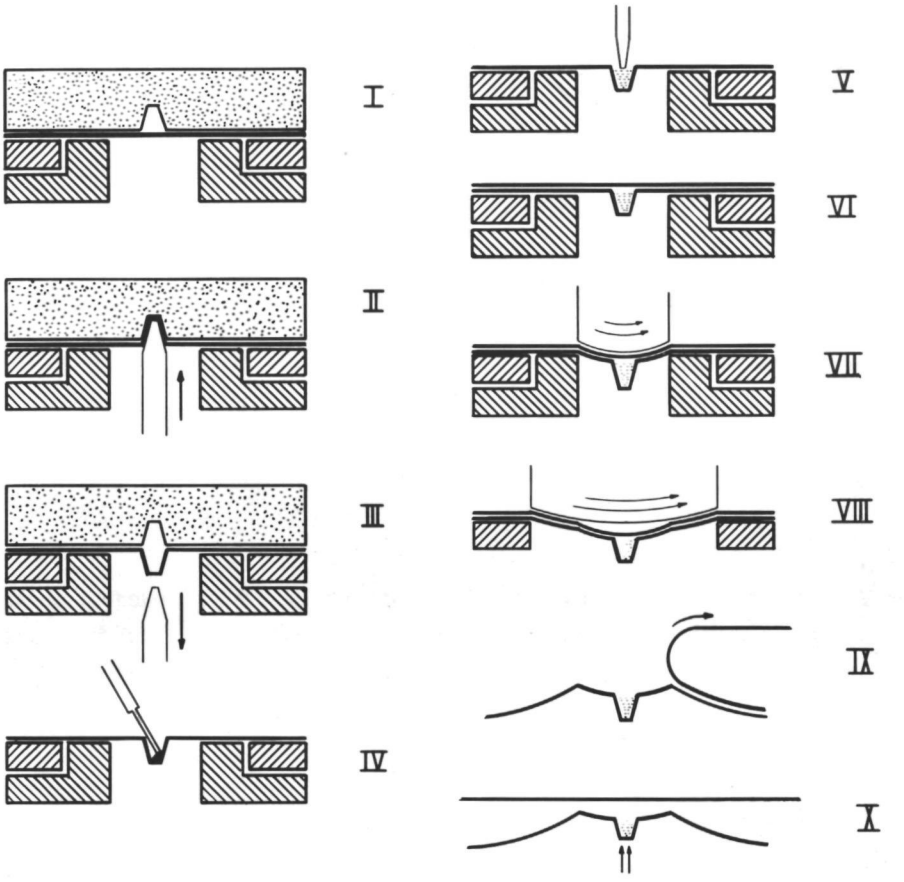

Figure 1: Incubation with lyophilized lymphocytes in parafilm microcuvettes(PMC, 3 µl). I, II and III: preparation of the PMC in a teflon mould. IV: PMC is filled with cell suspension which is subsequently frozen and lyophilized. V: PMC with lyophilized lymphocytes is filled with 3 µl incubation mixture. VI, VII and VIII: PMC is covered with a second strip of parafilm and double sealed with teflon pins on a stainless steel block. IX: after incubation in a water bath the second parafilm strip is peeled off. X: the contents of the PMC can be pressed out on chromatography paper or an aliquot can be taken and pipetted on thin-layer chromatography plates.

metabolites were co-chromatographed and after detection under UV light the spots were cut out and counted in a liquid scintillation counter (Packard, TriCarb B 2450). Analysis of the ADA reaction mixture was performed with *tert*-butanol(Merck-Schuchardt 82264), Darmstadt, F.R.G.): ethylmethylketone(Merck 9708):water:ammonia(Merck 5426) = 20:15:10:15 on PEI cellulose plates.

The adenosine kinase(AK; E.C. 2.7.1.20) assay was adapted from Meyskens and Williams(10). To the lyophilized lymphocytes 3 µl of a reaction mixture were added, containing 10 µM 8-^{14}C-adenosine, 1.5 mM ATP(Boehringer, Mannheim), 0.3 M trisodium-acetate/acetic acid(pH 5,7), 0.6 mM $MgCl_2$, 12.5 µM erythro-9-(2-hydroxy-3-nonyl)-adenine(EHNA), kindly supplied by Dr. H.A. Simmonds(Purine Laboratory, Guy's Hospital Medical School, London, UK) and 0.2%(v/v) triton X-100(Sigma T-6878). After incubation(1 hour at 37^0 C) separation of substrate and products was performed by means of thin-layer chromatography as described above for HGPRT.

Purine-5'nucleotidase(5'NT; E.C. 3.1.3.5) assay(8): to the lyophilized lymphocytes 3 µl of a reaction mixture were added, containing 0.05 M tris/HCl(pH 8.5), 0.02 M $MgCl_2$, 6.25 mM 2-glycerophosphate(Sigma G-6251), 0.6 mM 8-^{14}C-AMP(specific activity 56 mCi/mmol; RCA) and 0.2% (v/v) triton X-100. Incubation(3 hours at 37^0 C) was followed by separation of substrate and products as in the AK assay. Ecto-5'NT was assayed essentially according to Edwards et al.(4). In a total volume of 40 µl, 100,000 intact lymphocytes were incubated for 45 to 60 min. at 37^0 C. Activity estimation was carried out as described for 5'NT.

AMP deaminase(AMPD; E.C. 3.5.4.6) was assayed essentially according to Leech and Newsholme(9). In a mixture containing 60 mM Pipes(Merck 10220), 12 mM AMP(Merck 1428), 6 mM EDTA(Merck 8418), 30 mM KCl(Merck 4933), 2.5 mM dithiothreitol(Sigma D-8255), 180 µM 8-^{14}C-AMP(specific activity 56 mCi/mmol; RCA) and 0.2%(v/v) triton X-100, 6,000 lyophilized lymphocytes were incubated for 2 hours at 37^0 C.

The specific enzyme activity is calculated from the net product formed and is expressed in 10^{-9} moles/10^6 lymphocytes.hour. The total amount of radioactivity recovered after chromatography varies slightly for the individual incubations: this variation is due to micropipetting errors. To correct for this, the error in each individual measurement is calculated from the sum of product and remaining substrate. Thus, the product measured in counts per minute is not directly representative of the product actually formed by the enzyme reaction.

Table 1: Leukocyte Content at Different Stages of Lymphocyte Isolation

	Lymphocytes (%)	Monocytes (%)	Granulocytes (%)	LUC[1] (%)
Whole EDTA blood	28	6	61	5
After defibrination on glass beads	39	6	53	2
After filtration over nylon wool	91	1	6	2
After Ficoll-Isopaque density centrifugation	97	0	1	2

Hemalog D results are given. [1]LUC: large unstained cells; mainly lymphoid cells.

RESULTS

Lymphocyte Isolation

The composition of the various cell suspensions during lymphocyte

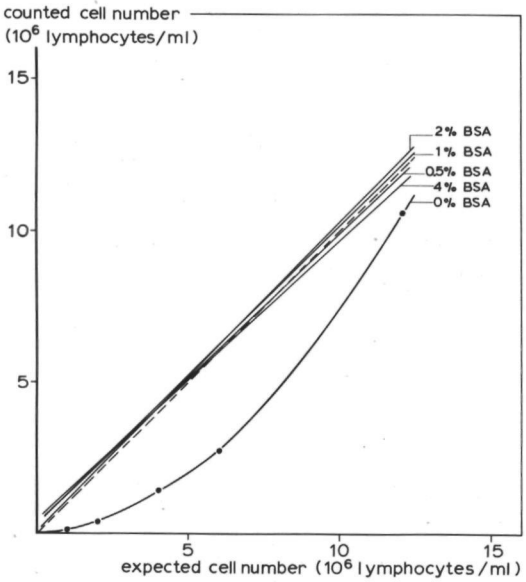

Figure 2: Relation between expected cell number, after dilution from a stock suspension and actually counted cell number. The BSA concentrations indicated represent the actual amounts of BSA present in the diluted cell suspensions. The broken line represents the position where the expected cell number equals the counted cell number.

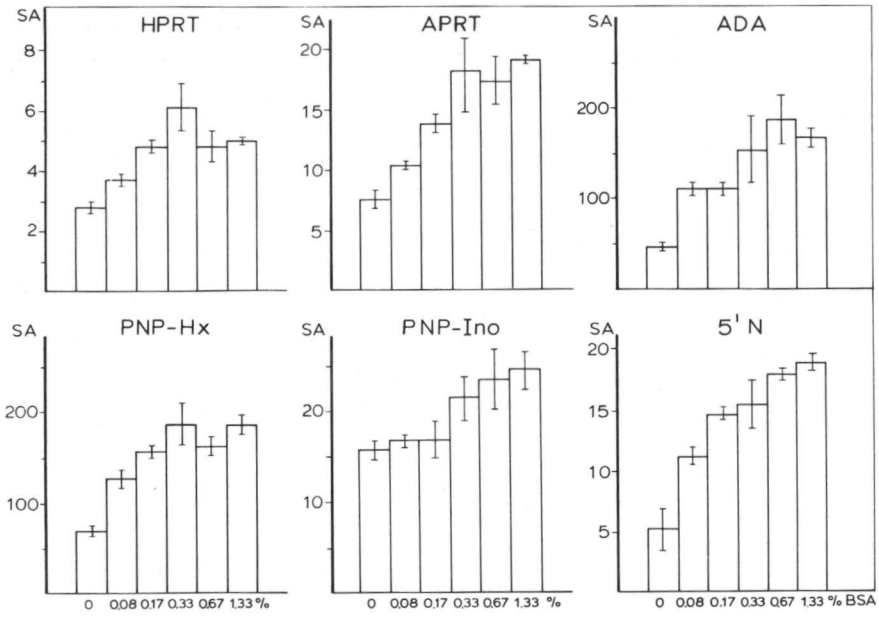

Figure 3: Effect of the BSA concentration on 6 different enzyme activities. The BSA concentrations indicated on the abscissa represent the final concentrations in the reaction mixtures, resulting from a six-fold dilution of the cell suspensions used. The vertical bars indicate the standard deviations of determinations carried out in quintuplicate. Specific enzyme activities(S.A.) are expressed in 10^{-9} moles/10^6 cells. hour.

purifications is shown in table 1. This procedure of de Pauw et al.(11) yields a fraction which contains over 97% lymphocytes with very low contamination of monocytes, granulocytes and large unstained cells(LUC).

Effects of Bovine Serum Albumin(BSA)

Lymphocyte suspensions isolated from peripheral blood normally contained more than 15 x 10^6 cells/ml. Before pipetting the cells into the PMC's, suspensions were prepared in the range of 1 x 10^6 to 12 x 10^6 cells/ml in polypropylene Eppendorf micro-test tubes. When 20 µl aliquots were taken and checked for the number of cells, a lower number of cells were found with the Coulter counter than expected on the basis of the dilution factor(fig. 2). The presence of BSA in the cell

Table 2: Reproducibility of the micro-assay for ADA and PNP, assayed in quintuplicate on the same sample. ADA assay: incubation with 1,000 cells for 0.5 hour at 37° C, input 60,000 cpm 8-^{14}C-adenosine(0.26 mM). PNP assay: incubation with 1,000 cells for 15 min. at 37° C, input 30,000 cpm 8-^{14}C-hypoxanthine(0.13 mM). For calculation of enzyme activities see under Materials and Methods.

	probe no.	net product formed (cpm)	enzyme activity (10^{-9} moles/10^6 cells.hour)
ADA	1	3.137	134
	2	5.702	139
	3	5.855	117
	4	4.781	125
	5	6.698	154
			mean ± s.d. 134 ± 14
PNP	1	5.550	306
	2	6.858	242
	3	7.983	292
	4	4.472	256
	5	6.515	247
			mean ± s.d. 269 ± 29

suspension gave a dilution curve, which was identical with that expected. In suspensions with low numbers of cells the differences between the expected cell concentration and the counted number were the most pronounced.

In the presence of BSA higher specific enzyme activities were observed(fig. 3). This increase was not due to chance enzyme activity present in the BSA, since complete incubation mixtures with BSA and without cells were used as blanks. The addition of BSA did not produce a significant rise in blank values. The purine enzyme activities in peripheral blood lymphocytes from a control individual showed highest activities in the presence of more than 0.33% BSA(w/v). For routine determinations a concentration of 0.67%(w/v) was used.

Reproducibility

Table 2 gives an example of ADA and PNP assays carried out in quintuplicate with lymphocytes from a healthy control subject. The standard deviations of both measurements is approximately 10% of the

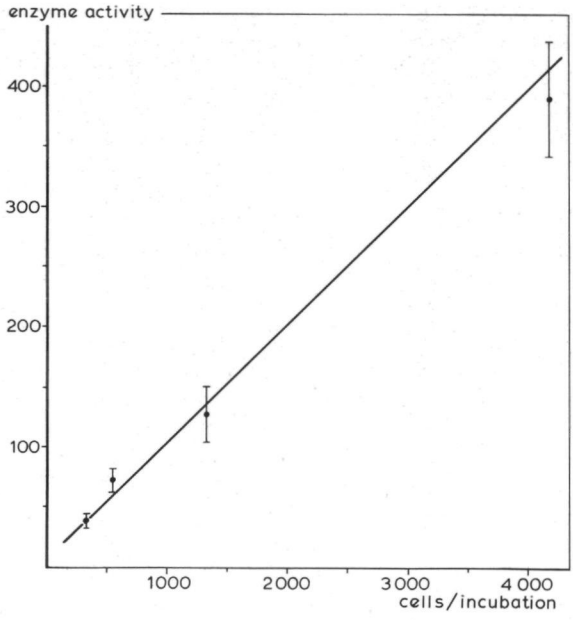

Figure 4: Relation between the number of lyophilized cells incubated and product formation in the adenosine deaminase reaction. Closed circles represent the mean activity of determinations carried out in quintuplicate. The vertical bars indicate the standard deviations. Enzyme activities are expressed in 10^{-9} moles/hour.

mean value. The reproducibility of the HGPRT, APRT, AK, 5'NT and AMPD assays was found to be at a comparable level(data not shown).

Fig. 4 shows the ADA activity as a function of the number of cells incubated. A linear relationship is seen up to at least 4,000 cells. The time curve for the ADA reaction is shown in fig. 5. Under the given incubation conditions the reaction was linear up to 3 hours at least. For routine determinations a cell number of 1,000 cells/incubation and an incubation time of 30 min. was chosen. Based on similar determinations of other enzyme activities, the individual assay conditions were chosen as summarized in table 3.

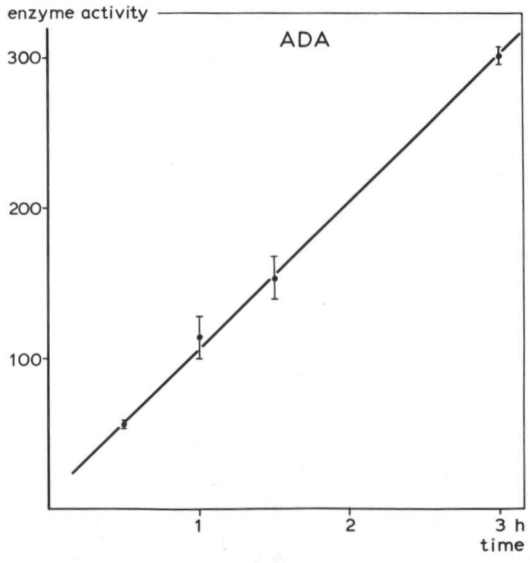

Figure 5: Time-curve for the adenosine deaminase(ADA) reaction. Closed circles represent the mean activity of determinations carried out in quintuplicate. The vertical bars indicate the standard deviation. Enzyme activities are expressed in 10^{-9} moles/10^{6} lymphocytes.

Ezyme Activities in Control Lymphocytes

Of the 7 different enzymes tested, PNP and AMPD displayed the highest specific activities(table 4). The activities of the salvage enzymes

Table 3: Micro-Assay Conditions

Enzyme	Substrate	Incubation time (min.)	Cells per incubation
HGPRT	hypoxanthine	90	6,000
APRT	adenine	90	3,000
ADA	adenosine	30	1,000
	deoxyadenosine	60	1,000
PNP	hypoxanthine	15	1,000
	inosine	90	3,000
AK	adenosine	60	6,000
5'NT	AMP	180	6,000
AMPD	AMP	120	6,000

Table 4: Purine Interconversion Enzymes in Lymphocytes of Healthy Controls(n=10)

Enzyme	Substrate	No BSA			0.67% BSA		
		Mean	s.d.	s.d. as % of mean	Mean	s.d.	s.d. as % of mean
HGPRT	hypoxanthine	2.95	0.72	24	6.27	0.84	13
APRT	adenine	8.93	1.56	17	14.7	1.7	12
ADA	adenosine	46.3	10.9	24	146	28	19
	deoxyadenosine	34.4	9.0	26	72.7	20.3	28
PNP	hypoxanthine	63.9	16.7	26	240	37	15
	inosine	13.0	1.8	14	24.8	3.5	14
AK	adenosine	0.74	0.47	64	9.67	3.57	37
5'NT	AMP	12.3	7.1	58	14.8	2.7	18
ecto-5'NT	AMP	NT[1]			6.67	1.46	22
AMPD	AMP	NT			359	54	15

[1]NT=not tested. Specific activities are expressed as 10^{-9} moles/10^{6} cells.hour

HGPRT and APRT were considerably lower. The activity of ADA with deoxyadenosine was about half that with adenosine. The PNP-catalyzed conversion of hypoxanthine to inosine was considerably more active than that in the opposite catabolic reaction(table 4). As compared with assays without BSA, all enzyme activities were higher in the presence of 0.67% BSA in the reaction mixture(table 4, fig. 3). In addition, the standard deviations of the mean values of almost all enzymes were smaller when BSA was used.

DISCUSSION

A relatively simple approach to ultramicrochemistry has been introduced for enzyme assays in lyophilized cultured fibroblasts, which makes use of fluorogenic substrates(5,6,7). These methods have also been applied to radiochemical assays(2,13,15). Biochemical and kinetic studies may be performed with this method, even at the "single cell" level(14,16).

A principal advantage of ultramicromethods is that a favourable signal:noise ratio can be obtained. The noise, or "blank", in an enzyme reaction is almost invariably due to the amount of substrate employed. The signal:noise ratio can be improved significantly by decreasing the incubation volume(lower blank value) and prolonging the incubation time (stronger signal). Additional advantages include:

1. Very limited amounts of material can be handled. The microtechnique described allows a range of biochemical studies to be performed on a given blood sample, which would have been impossible with conventional techniques. This is of special importance when lymphocyte subfractions are to be analyzed(18).
2. Because of the small incubation volumes(3 µl) only small amounts of substrates and reagents are needed, which helps to reduce the cost of large scale routine measurements. Moreover, the complete reaction mixture can be analyzed and no aliquots need to be taken.
3. Not only radioactive substrates can be used for enzyme assays in lymphocytes: the activity of a number of lysosomal hydrolases(e.g. hexosaminidase and acid phosphatase), can be readily and reliably determined by use of fluorogenic substrates(Van Laarhoven, unpublished results).

Before pipetting the lymphocytes into the PMC's, suspensions are prepared containing theoretically 1,000 to 6,000 cels per 0,5 µl aliquot. These suspensions are prepared in plastic Eppendorf reaction vessels. When aliqouts were taken for cell counting in a Coulter counter, fewer cells than expected are recovered. This effect is most significant in the range we normally use for our incubations(fig. 2). When BSA is included in the dilution medium, this effect is not seen; 0.5% BSA seems to be sufficient. We suspect that in the absence of BSA a certain number of cells stick to the wall of the polypropylene Eppendorf vessel. This effect is also seen when working with glassware. The smaller the absolute number of cells, the more pronounced the loss: e.g. in the case of 1×10^6 cells/ml the loss is 90% and in the case of 6×10^6 cells/ml the loss is 55%.

When enzyme activities are calculated on a per cell basis, it becomes evident that BSA also has a protective effect on all enzymes tested. This is illustrated in fig. 3. At a final concentration of 0.33% BSA(w/v) in the assay mixture maximum levels of activity were reached for almost all enzymes. The stabilizing effect was most pronounced in the case of ADA(a factor of 4.0; fig. 3). The enzymes are possibly protected from denaturation during both the lyophilization step and incubation. The benificial effect of BSA is also evident in the results in table 4; the range of control values in the presence of BSA is smaller as compared with the range observed when no BSA is added.

The reproducibility and reliability of the present microassays are comparable with conventional methods. This is the case not only with ADA and PNP(table 2), but also with the other enzymes tested. The kinetic analysis of enzyme reactions with only a few thousand cells(present study) is as feasible as conventional analysis with 2.5×10^5 cells(4) or 10^8 cells(3). The range of specific enzyme activities in control individuals obtained with this method is comparable with the range of conventional enzyme determinations published by others(3,12).

With the present micromethod a large number of enzyme activities can be measured in lymphocytes from a relatively small blood sample(5 to 10 ml). The methods are especially advantageous when subfractions of lymphocytes are to be studied and when the availability of cell material

is limited, e.g. in the case of children with lympho-proliferative disorders.

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chapter 4

ENZYMES OF PURINE INTERCONVERSIONS IN SUBFRACTIONS OF LYMPHOCYTES

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ABSTRACT

The present study was undertaken to investigate purine interconversion enzymes in lymphocyte subfractions in order to characterize purine metabolism. Thus we would like to contribute to the understanding of the relation between lymphocyte function and purine metabolism.

Lymphocytes were isolated from peripheral blood by nylon wool filtration and Ficoll-Isopaque density centrifugation. Separation of this lymphocyte fraction into a T cell and a non-T cell subfraction was carried out by E-rosette sedimentation. The activities of 6 purine interconversion enzymes(hypoxanthine-guanine phosphoribosyltransferase, adenine phosphoribosyltransferase, adenosine deaminase, purine nucleoside phosphorylase, adenosine kinase and purine-5'nucleotidase) in these subfractions were assayed by a newly developed micromethod. Along with similarities there were also differences found between the enzyme activities in T and non-T lymphocytes.

Mitogenic stimulation by phytohaemagglutinin and pokeweed mitogen was studied on purified lymphocyte fractions as well as on fractions contaminated with 20-30% non-lymphoid cells(by omitting the nylon wool filtration step from the isolation procedure). Phytohaemagglutinin had hardly any effect on the enzyme activities of both the pure lymphocyte fractions and the contaminated fractions. Pokeweed mitogen stimulation of contaminated lymphocyte fractions had a marked effect on the enzyme activities studied(e.g. up to 5 times for hypoxanthine-guanine phosphoribosyltransferase). The effect of pokeweed mitogen on pure fractions was less pronounced.

INTRODUCTION

Previously described micromethods for the determination of purine interconversion enzyme activitiesin lymphocytes(11) enabled us to analyse purine metabolism systematically in lymphocyte subfractions using a relatively small number of cells(500 to 5,000). A relation between purine interconversion defects and immune dysfunctions has been established(3, 4,5). The mechanism by which adenosine deaminase(ADA) deficiency leads to impairment of the B and T cell and purine nucleoside phosphorylase (PNP) deficiency leads to T cell dysfunction is not yet completely understood. A better understanding of purine interconversions in B and T cell subfractions might help to obtain a better view on B or T cell specificity in these immune diseases. One of the possibilities to achieve this might be a systematic enzymological analysis of purine metabolism in T and non-T lymphocytes. Nine purine enzyme activities were measured in T and non-T lymphocyte subpopulations using 500 to 5,000 cells per assay.

MATERIALS AND METHODS

Isolation of Lymphocyte Subfractions

Lymphocytes were isolated from peripheral blood by nylon wool filtration and Ficoll-Isopaque density centrifugation(2,11) and diluted to 2×10^6 cells/ml. Sheep erythrocytes were treated with neuraminidase (Behringwerke) and diluted in Earle's balanced salt solution(BSS) to 1×10^8 cells/ml. Equal volumes of lymphocytes and neuraminidase treated sheep erythrocytes were mixed and incubated at 37^0 C; after 15 min. the cells were spun down and E-rosettes were allowed to form(1 hour, 0^0 C). After a second Ficoll-Isopaque density centrifugation the non-rosetted non-T cells were collected from the interphase(7). The rosetted T cells were obtained from the pellet, after lyzing the sheep erythrocytes with a solution containing 155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM disodium EDTA (pH 7.4).

"Micro" Assays with Lymphocytes

Enzyme incubations were carried out according to Van Laarhoven et al.(11). The following enzymes were assayed: ADA with adenosine and deoxyadenosine as a substrate, hypoxanthine-guanine phosphoribosyltransferase(HGPRT) with both hypoxanthine and guanine as a substrate, adenine phosphoribosyltransferase(APRT), adenosine kinase(AK) and purine-5'nucleotidase(5'NT) as a substrate.

Mitogenic Stimulation of Unfractionated Lymphocyte Preparations

The isolated lymphocytes were resuspended in tris-buffered minimal essential medium(MEM, Gibco F-14; pH 7.4) containing 20% human A^+ serum. After dilution of the cells to a concentration of 3×10^5 cells/ml, portions of 1 ml each were divided into sterile tubes(Nunc nr. 1090). To test phytohaemagglutinin(PHA) stimulation, an equal number of tubes was cultured for 3 days at 37^0 C with and without addition of 0.5 µl PHA-P (Difco 3110-57). In similar experiments 25 µl/ml pokeweed mitogen(PWM; Gibco) was added to a final concentration of 25 µgr/ml. Cells were cultured for 7 days at 37^0 C. Twentyfour hours before harvesting 0.5 µCi/ml ^{3}H-thymidine(specific activity 24 Ci/mmol; Radiochemical Centre Amersham, UK) was added to the cultures. The cells were harvested by filtration on glass fiber filters(Millipore AP 2002500). After incubation of the filters(½ hour, 20^0 C) in liquid scintillation counting vials with 0.5 ml NCS tissue solubilizer(Radiochemical Centre Amersham, UK) diluted(1:3) with counting fluid(MI 92, Packard), 10 ml MI 92 was added, containing 1% acetic acid(v/v). For the enzymatic assays the cells were collected after 3 days(PHA) and 7 days(PWM) by centrifugation(600 g, 10 min.), resuspended in 0.9% NaCl(w/v) and lyophilized as described above.

RESULTS AND DISCUSSION

The activities of HGPRT and PNP in T and non-T subfractions were in the same range(table 1). Mean activities of APRT, AK and 5'NT were higher in T cells as compared to non-T cells. One enzyme was more active in non-T than in T cells: ADA. This was found with both deoxyadenosine and

Table 1: Purine Interconversion Enzymes in Lymphocyte Subfractions

Enzyme	Substrate	T fraction	non-T fraction	n
HGPRT	hypoxanthine	3.24 ± 1.01	3.14 ± 1.68	10
	guanine	5.88 ± 2.28	5.66 ± 2.54	10
APRT	adenine	9.82 ± 5.88	4.48 ± 2.54	10
ADA	adenosine	61.67 ± 27.73	91.62 ± 49.01	10
	deoxyadenosine	35.53 ± 13.18	63.72 ± 38.65	9
PNP	hypoxanthine	74.45 ± 43.44	66.89 ± 34.21	10
	inosine	15.56 ± 4.60	12.59 ± 4.24	10
AK	adenosine	0.88 ± 0.63	0.47 ± 0.31	8
5'NT	AMP	12.31 ± 7.08	3.29 ± 2.46	7

Activities ± standard deviation, calculated from a group of healthy individuals and expressed as 10^{-9} moles product formed/10^{6} lymphocytes .hour.

adenosine as a substrate(table 1). These latter findings are different from those reported by Huang et al.(6) who found that ADA activities of complement-receptor negative(T) cells were approximately 10 times higher than those of complement-recetor positive(B) cells. Differences in ADA activity in B and T cells have also been reported by several other groups (9,10). Purine nucleoside phosphorylase was shown to display similar activities in T and non-T cells. A number of investigators however, has reported that PNP activity is higher in T cells. PNP has even been suggested as a T cell marker on the basis of histochemical findings(1). These inconsistencies might be due to the differences of isolation procedures used. Our isolation procedure is based on the E-rosette forming capacity. Other workers made use of other immunological markers such as complement-receptors(6). Moreover, our subfractions are hardly contaminated(<3%) with monocytes or granulocytes. Such data are not available from other reports(1,6). Tritsch and Minowada(9) compared leukemic T cell lines with normal B cell lines and reported higher ADA activities in malignant T cell lines. To our knowledge there are no reports on HGPRT, APRT, AK and 5'NT activities in lymphocyte subfractions.

As can be seen in table 1 there exists a rather wide range of enzyme activities in healthy controls. This is due to individual variation and not to methodological errors(11). In addition, in a given individual enzyme activity ratios are constant, e.g. PNP activities are consistently higher than APRT activities(table 1).

Table 2: Effect of Culture Time on Purine Interconversion Enzymes in Unfractionated Lymphocytes in the Absence of Mitogens

Enzyme	Culture Time					
	0 days		3 days		7 days	
	P[1]	C[2]	P	C	P	C
HGPRT	100% (4.5)[3]	100% (5.7)	68%	83%	39%	50%
ADA	100% (99)	100% (88)	70%	74%	33%	33%
PNP[4]	100% (12.8)	100% (15.7)	95%	76%	77%	67%
5'NT	100% (19.7)	100% (20.0)	54%	77%	91%	112%

[1]"Pure" fractions(P) were isolated as described in materials and methods(contamination with non-lymphoid leukocytes less than 3%).
[2]"Contaminated" fractions(C) are isolated in the same way as "pure" fractions but the nylon wool filtration step is omitted(contamination: 2 to 3% monocytes, 20 to 30% granulocytes). [3]Values in parentheses represent the absolute enzyme activities in 10^{-9} moles/10^6 cells.hour.
[4]As a substrate for the PNP assay, 8-^{14}C-inosine was used.

Mitogenic Stimulation of Unfractionated Lymphocytes

In order to test the effect of the mitogens PHA and PWM on unfractionated lymphocytes, two different lymphocyte preparations were employed. A "pure" fraction was obtained by the complete procedure as described under materials and methods. A "contaminated" fraction was obtained by following the same procedure, but omitting the nylon wool filtration step. The "pure" preparation contained more than 97% lymphocytes, 1% monocytes and 1% granulocytes(according to Hemalog D determinations). The "contaminated" fraction contained 72% lymphocytes, 2% monocytes and 25% granulocytes.

In order to be able to assess the real stimulation index of purine enzyme activities an experiment was performed in which, in the absence of mitogens, the activity of HGPRT, ADA, PNP, and 5'NT was tested after 3 days(culture time for PHA stimulation test) and after 7 days(culture time for PWM stimulation test). Three out of four enzymes tested(HGPRT, ADA and PNP) showed a decrease in activity during culturing, whereas one enzyme(5'NT) displayed a lower activity after 3 days, but after 7 days the activities were again around the original level. It was concluded that the results of the PHA stimulation experiments should be related to the control values obtained after 3 days of culturing in the absence

Table 3: Effect of Mitogens on ^{3}H-Thymidine Incorporation and on Purine Interconversion Enzymes in Unfractionated Lymphocytes

	PHA(3 days)		PWM(7 days)	
	P[1]	C[2]	P	C
^{3}H-Thymidine Incorporation Index	10[3]	120	25	25
HGPRT	1.3	2.5	3.6	4.6
ADA	1.0	1.0	2.4	2.3
PNP[4]	1.1	1.3	1.5	1.6
5'NT	1.2	1.0	1.1	3.4

[1]"Pure" fractions. [2]"Contaminated" fractions. [3]The values given represent the ratio between the activity measured with and without mitogen; for PHA stimulation the values after 3 days of culturing were used, and for PWM stimulation the values after 7 days. [4]As a substrate for the PNP assay, 8-^{14}C-inosine was used.

of this mitogen. The same was done in the PWM experiments(culturing time 7 days).

The "pure" lymphocytes were hardly stimulated by PHA as judged from the ^{3}H-thymidine incorporation. The response of the "contaminated" lymphocytes to PHA was considerably higher(Table 3). PWM stimulated both the "pure" and the "contaminated" cells to the same extent. The activities of the purine enzymes tested remained unchanged after PHA stimulation in both lymphocyte preparations with the possible exception of HGPRT in the "contaminated" lymphocytes. The HGPRT activities of "pure" and "contaminated" lymphocytes after 7 days of PWM stimulation were clearly increased. A less pronounced rise was observed for ADA, whereas PNP activity did not show significant stimulation. The marked rise after PWM stimulation of 5'NT activity in "contaminated" lymphocytes as compared to "pure" cells was ascribed to contaminating cells, especially granulocytes. Our results concerning the effect of culture time on lymphocytic HGPRT, PNP, ADA and 5'NT activities in the absence of the mitogens are essentially in agreement with data reported by Raivio and Hovi(8) on T cell enriched lymphocyte preparations. On the other hand, our data on ADA after PHA stimulation do not show the increase in activity as described by the latter workers. It should be emphasized however, that the present study was carried out under other experimental

conditions. This makes comparison with other reports(6,9,10) difficult. On the basis of findings with PHA stimulated intact lymphocytes(8) increased levels of purine phosphoribosyltransferase activities might be expected. This is indeed the case with HGPRT in the "contaminated" cells, but not in "pure" cells. As judged from the ^{3}H-thymidine incorporation, the "contaminated" cell population was synthetizing DNA more actively than the "pure" lymphocytes after PHA stimulation. Therefore, it seems that the rate of purine reutilization is associated with the rate of DNA synthesis. This phenomenon is also observed in PWM stimulated "pure" and "contaminated" cells. Although satisfying explanations for our findings cannot yet be given, the differential responses to PHA and PWM seem to point to differences in purine metabolism in PHA and PWM responsive cells.

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chapter 5

PURINE INTERCONVERSION PATHWAYS IN T, B, T_γ AND T-T_γ CELLS FROM HUMAN PERIPHERAL BLOOD

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ABSTRACT

Human peripheral blood lymphocytes(PBL) were prepared by depleting peripheral blood mononuclear cells(PBMC) of adherent cells(AC) and separated in T and B cell enriched fractions. T cells were subdivided in $T\gamma$ and T-$T\gamma$ cells. Eight enzymes involved in purine metabolism were assayed in the fractions mentioned above. No significant differences in enzyme activities were noticed between the PBMC and the AC depleted PBL fractions. In the B cell enriched fraction the activities of hypoxanthine-guanine phosphoribosyltransferase(HGPRT), purine nucleoside phosphorylase(PNP), ecto-5'nucleotidase(5'NT), adenosine kinase(AK), AMP deaminase(AMPD) and adenylate kinase(AdKin) were found to be at least twice as high as in the T cell enriched fraction. Strong indications were obtained that platelet contamination in some of the isolated fractions might at least in part influence the noted differences in enzyme activities. When comparing $T\gamma$ and T-$T\gamma$ cells, higher specific activities of PNP and adenine phosphoribosyltransferase(APRT) were found in T-$T\gamma$ cells, whereas AdKin activity was found to be higher in the $T\gamma$ fraction. Due to the differences in enzymatic make-up $T\gamma$ cells might be more sensitive to purine nucleoside intoxication than T-$T\gamma$ cells.

INTRODUCTION

In the last decade it has become clear, that a number of immunological diseases are related to imbalances of purine metabolism in lymphocyte subpopulations(7,12,26). Information regarding the enzymatic make-up and metabolic characteristics of lymphocyte subpopulations, both in healthy individuals and in patients, might contribute to a better diagnosis of immunological diseases and be of importance for the development of immunoregulatory drugs. Not only T and B cell subpopulations, but also T and B cell subsets should be included in such studies.

Different activity levels of enzymes involved in purine metabolism have been described in subpopulations of human peripheral blood lymphocytes(1,10,14,17,21,22). With respect to T and B lymphocytes conflicting data were reported on adenosine deaminase(ADA; E.C. 3.5.4.4), purine nucleoside phosphorylase(PNP; E.C. 2.4.2.1) and ecto-5'nucleotidase(5'NT; E.C. 3.1.3.5) activities. To our knowledge no systematic studies on these enzyme activities in T cell subsets have been published.

In the present study a recently described micromethod(23) has been used to investigate the activities of purine interconversion enzymes (fig. 1) in various human peripheral blood lymphocyte subpopulations, including T and B cells and the T cell subset bearing surface receptors for the Fc portion of IgG(T_γ cells). These lymphocyte fractions have been assayed for ADA, PNP, 5'NT, hypoxanthine-guanine phosphoribosyltransferase(HGPRT; E.C. 2.4.2.8), adenine phosphoribosyltransferase (APRT; E.C. 2.4.2.7), adenosine kinase(AK; E.C. 2.7.1.20), AMP deaminase (AMPD; E.C. 3.5.4.6) and adenylate kinase(AdKin; E.C. 2.7.4.3) activities.

MATERIALS AND METHODS

Lymphocyte Preparation

Peripheral blood mononuclear cells(PBMC) from 5 healthy volunteers were prepared by centrifugation of heparinized blood, diluted with two volumes of Hank's balanced salt solution, on Ficoll-Metrizoate(1.077 gr/ml; 21). For removal of adherent cells(AC), PBMC were resuspended in

in Hepes buffered RPMI 1640(Flow Labs., Rockville, MD) supplemented with 20%(v/v) foetal calf serum(FCS; Microbiological Ass., Bethesda, MD) at a cellular concentration of 2 x 10^7 cells/ml. This suspension was incubated in a plastic Petri dish(Falcon 3045) for 1 hour at 37^0 C(6 ml/dish).

Peripheral blood lymphocytes(PBL = PBMC minus AC) were separated in T and B lymphocyte fractions by density sedimentation of spontaneous rosettes formed by T lymphocytes and sheep erythrocytes treated with 2-amino-ethyl-isothiouronium bromide(AET; 18). T cells were further separated into T_γ enriched and T_γ depleted($T\text{-}T_\gamma$) fractions according to Moretta et al.(13) by rosetting with ox erythrocytes coated with rabbit anti-bovine eryhtrocytes IgG(Nordic Lab.). Contaminating erythrocytes in each lymphocyte preparation were lyzed by incubation with 0.16 M ammonium chloride in a 0.01 M potassium bicarbonate, 0.1 mM EDTA buffer. Identification of monocytes and polynuclear cells was performed by the peroxidase reaction(16). The B cells were identified by membrane fluorescence(16), employing FITC/swine anti-human immunoglobulin serum (Nordic Lab.). T cells were counted as cells forming rosettes with AET treated sheep erythrocytes.

Enzyme Assays

Enzyme assays were carried out essentially according to a previously described radiochemical micromethod(23). HGPRT and APRT were assayed by measuring the conversion of ^{14}C labeled hypoxanthine and adenine to the corresponding mononucleotides IMP and AMP, respectively. ADA activity was assayed by following the deamination of adenosine to inosine. PNP was assayed in the anabolic reaction: from hypoxanthine to inosine. AK activity was determined by measuring the conversion of adenosine to AMP. The α,β-methyleneadenosine-5'diphosphate(AOPCP) inhibitable degradation of AMP to adenosine was taken as 5'NT activity(5). AMPD was assayed by measuring the conversion of AMP to IMP(23). AdKin was assayed according to Leech and Newsholme(11) adapting their system to our micromethod.

Table 1: Characterization of the Lymphocyte Preparations

Marker	PBMC[1], total	PBL[2], AC[3] depleted	T	B
E_{aet}[4]	82.4 ± 2.7	81.2 ± 2.4	94.0 ± 1.9	NT[5]
sIg[6]	14.4 ± 5.3	11.3 ± 3.4	0.2 ± 0.4	78.8 ± 5.4
Peroxidase[7]	13.5 ± 1.0	4.0 ± 1.4	0.5 ± 0.6	6.5 ± 2.5

The figures indicate the percentage of cells(mean ± s.d.; n=5) from a particular fraction possessing the investigated marker. [1]Peripheral blood mononuclear cells. [2]Peripheral blood lymphocytes. [3]Adherent cells. [4]Cells rosetting with AET treated sheep erythrocytes. [5]Not tested. [6]Surface immunoglobulin bearing cells. [7]Peroxidase positive cells.

Statistical Analysis

The differences in enzymatic activities between the various cell populations were analyzed by a two-tailed t-test of Student.

RESULTS

Characterization of the Lymphocyte Fractions

The isolated PBMC from 5 healthy donors contained 82.4% E_{aet} positive cells, 14.4% sIg bearing cells and 13.5% peroxidase positive cells (mean values; table 1). After adherence of this cell population to plastic Petri dishes the number of E_{aet} and sIg positive cells hardly showed any change, whereas the percentage of peroxidase stained cells significantly decreased. These AC depleted PBL were used for the separation of T and B subpopulations. After E_{aet} rosette depletion the T cell pellet contained 94.0% E_{aet} positive cells, but virtually no sIg bearing cells and no peroxidase stained cells. The T depleted preparations contained 78.8% sIg bearing cells and 6.5% peroxidase positive cells (table 1).

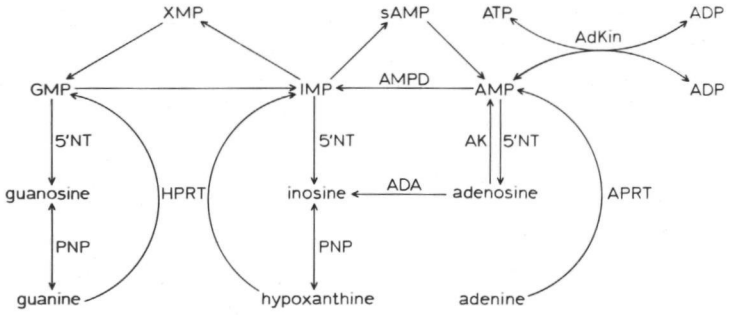

Figure 1: A simplified scheme of purine interconversion pathways. List of the enzymes and their abbreviations: ADA-adenosine deaminase; AdKin-adenylate kinase; AK-adenosine kinase; AMPD-AMP deaminase; APRT-adenine phosphoribosyltransferase; HGPRT-hypoxanthine-guanine phosphoribosyltransferase; 5'NT-purine-5'nucleotidase; PNP-purine nucleoside phosphorylase.

Enzyme Activities

The results of the enzyme determinations in the various subfractions are summarized in table 2. No statistically significant differences of the specific activities were noticed between the unfractionated PBMC and the AC depleted preparations of PBL(table 2).

When comparing the T and B cell subpopulations a significant difference in the activities of HGPRT($p<0.01$) and 5'NT($p<0.05$) was seen. The activities of these enzymes were twice as high in the B cell population as compared to the T cell population. The differences in activities of PNP($p<0.001$), AK($p<0.01$), AMPD($p<0.02$) and AdKin($p<0.001$) between the T and B cell subpopulations were also found to be significant. In B cell preparations the activities were about a threefold higher when compared to T cells.

In the T cell subsets, $T\gamma$ and T-$T\gamma$, no significant differences could be found in HGPRT, ADA, 5'NT, AdKin and AMPD activities. A significant difference however, was found in the PNP activity in these subsets. PNP activity was 43% higher in T-$T\gamma$ cells than it was in $T\gamma$ cells($p<0.05$). APRT activity was found to be 76% higher in T-$T\gamma$ cells than in $T\gamma$ cells ($p<0.05$). AK activity was twice as high in T-$T\gamma$ cells as compared to $T\gamma$ cells($p<0.02$). On the other hand, though not significant($p>0.05$), AdKin

Table 2: Purine Enzyme Activities in Fractionated Human Peripheral Blood Mononuclear Cells

Enzyme	PBMC[1] total	PBL[2], AC[3] depleted	T	B	Tγ	T-Tγ
HGPRT	12.5 ± 3.4	9.0 ± 2.2	5.7 ± 0.7	11.0 ± 3.2	5.1 ± 1.7	5.0 ± 1.2
APRT	13.8 ± 3.3	12.3 ± 3.3	10.8 ± 2.8	11.4 ± 3.9	6.1 ± 2.7	10.8 ± 3.2
ADA	92.7 ± 21.1	104 ± 23	99.2 ± 25.8	129 ± 53	131 ± 114	104 ± 3.2
PNP	468 ± 103	390 ± 120	159 ± 19	443 ± 122	104 ± 33	149 ± 26
AK	8.8 ± 3.2	7.1 ± 2.2	4.8 ± 1.1	13.0 ± 5.2	2.7 ± 1.1	4.6 ± 0.8
5'NT	8.3 ± 3.8	8.5 ± 4.8	6.5 ± 2.7	13.2 ± 7.4	4.3 ± 3.1	7.0 ± 1.5
AMPD	314 ± 145	296 ± 169	162 ± 74	362 ± 135	93 ± 73	145 ± 85
AdKin	921 ± 321	734 ± 331	221 ± 43	781 ± 304	395 ± 226	203 ± 43

Enzyme activities are expressed in 10^{-9} moles/10^{6} cells.hour(n=5). The mean specific activities are given ± standard deviations. [1]Peripheral blood mononuclear cells. [2]Peripheral blood lymphocytes. [3]Adherent cells. The significant differences($p<0.05$) are underlined.

was nearly twice as high in the T_γ cells as in the T-T_γ cells.

DISCUSSION

The present results show no significant differences in enzyme activity levels of unfractionated PBMC and AC depleted PBL(table 2). A possible explanation for these findings is that the enzymatic activities in the peroxidase positive cells do not differ much from those of purified lymphocytes(4,15). Another, more likely, possibility is that, due to the relatively small contamination of peroxidase positive cells, no significant changes of enzymatic activities are seen after removal of these cells(mainly monocytes). According to Edwards et al.(6) the 5'NT activity in PBL is 21.9 10^{-9} moles/10^6 cells.hour. In peripheral monocytes 5'NT activity is 4.2 10^{-9} moles/10^6 cells.hour. Extrapolating these data to our cell preparations, this would mean that our AC contaminated PBMC fraction would have a 5'NT activity of 19.5 10^{-9} moles/10^6 cells.hour. Our AC depleted PBL fraction would have a 5'NT activity of 21.2 10^{-9} moles/10^6 cells.hour. Such differences are not readily detected with the present assay methods.

Our data on 5'NT activities in B and T enriched fractions are consistent with some earlier reports(17,20) and at variance with others including ours(6,22). In our earlier studies different methods were used both for the lymphocyte isolation and for the 5'NT measurement. In the present study we used heparin as anti-coagulant and the AC were removed by adherence to plastic; in our previous work lymphocytes were purified from defibrinated blood and were filtered through a nylon wool column (3,22). The present procedure led to cellular preparations contaminated with platelets, whereas the other method led to platelet-free preparations. Still more relevant is our observation that B lymphocyte preparations obtained after rosette forming cell depletion were much more enriched in platelets than the T lymphocytes. When comparing the enzymatic activities between two lymphocyte preparations, it is essential to assess their platelet contamination since thrombocytes show considerable PNP and AdKin activities. In earlier AC depleted PBL preparations that were essentially free of platelets(as a consequence of defibrination with glass beads) mean PNP activity was 209 10^{-9} moles/10^6 cells.hour.

AdKin activity was 265 10^{-9} moles/10^6 cells.hour(24). In table 2(platelet contaminated, AC depleted PBL) these activities were 390 and 734 10^{-9} moles/10^6 cells.hour, respectively. The other enzymes showed comparable activities in platelet free and platelet contaminated PBL(24).

This leads to the conclusion that the higher AdKin and PNP activities in the B cell fractions, at least in part might be ascribed to the platelet contamination. With respect to the other enzymes, the differences in activity levels do not seem to be due to contaminating cells. From the foregoing it is clear that when comparing enzymatic activities in lymphoid cell subfractions it is necessary to carefully characterize the various fractions, in order to account for the different isolation procedures.

Differences of ADA activity in T and B lymphocytes do not seem to be significant(14,21,22). Our present data confirm these findings. Only Huang et al.(10) have found a considerable higher ADA activity in cells bearing C_3 receptors(B cells) as compared to cells which lacked this receptor(T cells).

The statistically significant differences found in APRT, PNP and AK activities in $T\gamma$ and T-$T\gamma$ cells cannot be obscured by the presence of platelets, since the latter are removed in the first E-rosette depletion step. The lower activity of PNP in $T\gamma$ cells seems inconsistent with the data of Levinson et al.(12). These authors found in T lymphocytes from patients with systemic lupus erythematosus(SLE) a decreased activity of PNP as compared to T cells from control individuals. In SLE decreased numbers of T suppressor cells($T\gamma$ cells) have been reported(8). From our finding, that $T\gamma$ cells have lower PNP activity than T-$T\gamma$ cells, it would be expected that in patients with relatively few $T\gamma$ cells, the PNP activity in total T lymphocytes should increase.

The present results on $T\gamma$ and T-$T\gamma$ cells suggest that $T\gamma$ cells might be more sensitive to purine nucleoside intoxication as compared to the T-$T\gamma$ cells. As a consequence of a higher capacity for nucleotide triphosphate synthesis(AdKin) and a lower breakdown capacity for nucleotides (5'NT), deoxyribonucleotides may more easily be accumulated. Deoxyribonucleotides(e.g. deoxyATP) are well known inhibitors of ribonucleotide

reductase, and may thus lead to impairment of DNA synthesis(19). This selective sensitivity was illustrated in experiments where cells were stimulated with concanavalin A and phytohaemagglutinin under ADA deficient conditions, The phytohaemagglutinin stimulated cells(mainly $T\mu$ cells; 9) were much less sensitive to adenosine and deoxyadenosine intoxication than concanavalin A stimulated cells(both $T\mu$ as well as $T\gamma$ cells; 9,25).

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chapter 6

ENZYMES OF PURINE METABOLISM AS DIAGNOSTIC TOOLS IN ACUTE LYMPHOBLASTIC LEUKEMIA

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ABSTRACT

The purpose of the present study was to investigate whether certain purine enzymes might be useful as differentiation markers in acute lymphoblastic leukemia. A micromethod has been developed for radiochemical assays of adenosine deaminase, purine nucleoside phosphorylase and 5'nucleotidase. In T type acute lymphoblastic leukemia hardly detectable levels of 5'nucleotidase were consistently observed in lymphoid cells derived from bone marrow. Adenosine deaminase showed very high activities as compared to normal T lymphocytes, whereas purine nucleoside phosphorylase levels were significantly lower than those in normal cells. In nonBnonT type acute lymphoblastic leukemia no deviant 5'nucleotidase activities were observed. Adenosine deaminase activity however, was three-fold higher as compared to control values. The enzymes studied are indeed useful as biochemical markers. In addition, attractive possibilities for enzyme directed chemotherapy might be opened up by taking advantage of the differences in enzymatic make-up in various leukemic and normal cells.

Enzymes of purine nucleotide synthesis in lymphoid cells from patients with acute lymphoblastic leukemia(ALL) have been studied by a number of investigators. Scholar and Calabresi(10) reported that activities of several enzymes of purine metabolism in leukemic lymphoid cells were changed as compared to activities in normal lymphocytes.

Elevated levels of adenosine deaminase(ADA; E.C. 3.5.4.4) were found in lymphoid cells from patients with ALL. In T-ALL ADA activities were found to be seven-fold(11) or even fourty seven-fold(5) higher than in normal lymphocytes. In contrast, ADA levels in nonBnonT-ALL were only slightly elevated, two to four-fold(5).

Purine nucleoside phosphorylase(PNP; E.C. 2.4.2.1) was reported to be decreased only in T-ALL(2). In normal mononuclear cells the median activity was 79 units, whereas in T-ALL cells the median was significantly lower(38 units).

In T-ALL an inverse linear relation has been reported between 5'nucleotidase(5'NT; E.C. 3.1.3.5) activity and the number of E-rosette positive cells. The mean 5'NT activity in T lymphocytes was significantly lower as compared to normal lymphocytes and null lymphoblasts(9).

A recently described micromethod(13) enabled us to assay nine different enzyme activities of the purine interconversion pathway in lymphoid cells from patients with ALL. Activities of ADA, PNP, assayed in the anabolic direction, 5'NT assayed both with intact cells(ecto-enzyme) and with lyophilized cells(total 5'NT activity, hypoxanthine-guanine phosphoribosyltransferase(HGPRT; E.C. 2.4.2.8), adenine phosphoribosyltransferase(APRT; E.C. 2.4.2.7), adenosine kinase(AK; E.C. 2.7.1.20), adenylate kinase(AdKin; E.C. 2.7.4.3) and AMP deaminase(AMPD; E.C. 3.5.4.6) were assayed in lymphoid cells from 20 controls, 24 patients with nonBnonT-ALL and 12 patients with T-ALL.

MATERIALS AND METHODS

Patients

At the time of diagnosis, 36 chidren with ALL(aged between 1 and 14 years) were investigated. In all cases the diagnosis was obtained on bone marrow aspirates and confirmed at the Reference Centre of the Dutch Working Group on Leukemia in Children(SNWLK), The Hague, The Netherlands.

Immunological classification of ALL

The immunological classification was performed only in almost completely homogeneous(>95%) leukemic cell populations from bone marrow and/or peripheral blood. The leukemic cells were isolated by the Ficoll-Isopaque gradient centrifugation method as described previously(1). Sheep erythrocyte(E) rosettes were used as a standard T cell marker(1). If more than 20% of the leukemic cell population formed E-rosettes at 4^0 C, the diagnosis T cell-ALL was made. As a criterion for B cell-ALL the presence of more than 20% surface immunoglobulin(sIg) bearing cells was used. Immunofluorescence studies were performed using tetramethyl rhodamine isothiocyanate(TRITC)-labeled anti-human Ig(1). Leukemic cell populations that did not meet the criteria mentioned above were classified as nonBnonT-ALL. As antisera against the common-ALL antigen could not yet be used in all cases studied, the last subgroup of ALL could not be subdivided(7) into "common-ALL" and "unclassified-ALL" according to the Greaves' nomenclature(7).

Enzyme Assays

The nine enzymes indicated previously, were assayed with radioactive substrates, using 1,000 to 6,000 cells per assay, according to the micromethod published earlier(13). AdKin was assayed according to Leech and Newsholme(8) adapting their system to our micromethod. Lymphoid cells from controls were isolated from peripheral blood(13), whereas in the case of patients the cells were taken from peripheral blood and/or bone marrow(see Immunological Classification).

Table 1: Purine Enzymes in Lymphoid Cells from Controls and ALL-Patients

Enzyme	Controls (n=20)	nonBnonT-ALL (n=24)	T-ALL (n=12)
HGPRT	6.9 ± 3.3[1]	13.8 ± 7.1	11.4 ± 5.1
APRT	13.6 ± 4.2	10.2 ± 5.9	6.9 ± 2.6
ADA	134 ± 46	491 ± 333	1069 ± 467
PNP	209 ± 70	200 ± 97	124 ± 69
5'NT	18.4 ± 11.7	25.9 ± 23.1	1.0 ± 1.8
ecto-5'NT	7.6 ± 3.0	8.3 ± 7.7	0.8 ± 0.7
AK	7.9 ± 3.6	6.8 ± 4.4	6.3 ± 2.6
AMPD	267 ± 106	314 ± 347	151 ± 120
AdKin	265 ± 183	791 ± 411	283 ± 230

[1]Mean specific activities ± standard deviations in 10^{-9} moles/10^6 cells. hour.

Statistical Evaluation

Statistical evaluation was performed using the two tailed Student t-test for grouped values with separate variance estimate.

RESULTS

In table 1 the activities of nine different enzymes involved in

Table 2: Statistical Evaluation of Purine Enzyme Activities in Control Lymphocytes vs. Leukemic Cells

Enzyme	nonBnonT-ALL	T-ALL
HGPRT	↑↑[1] ($p<0.001$)	↑[2] ($p<0.02$)
APRT	↓[3] ($p<0.05$)	↓↓[4] ($p<0.001$)
ADA	↑↑ ($p<0.001$)	↑↑ ($p<0.001$)
PNP	n.s.[5]	↓ ($p<0.01$)
5'NT	n.s.	↓↓ ($p<0.001$)
ecto-5'NT	n.s.	↓↓ ($p<0.001$)
AK	n.s.	n.s.
AMPD	n.s.	↓ ($p<0.02$)
AdKin	↑ ($p<0.01$)	n.s.

[1]↑↑Highly significant increased activity in leukemic cells. [2]↑Significantly increased activity in leukemic cells. [3]↓Significantly decreased activity in leukemic cells. [4]↓↓Highly significant decreased activity in leukemic cells. [5]n.s. No significant difference.

purine interconversions are shown. Lymphoid cells isolated from normal healthy controls, patients with nonBnonT-ALL and from patients with T-ALL were used. Statistical evaluation of these data revealed that when comparing enzyme activities in control lymphoid cells with enzyme activities in nonBnonT-ALL lymphoid cells, HGPRT, ADA and AdKin values were higher in nonBnonT-ALL than in controls, whereas APRT showed a lower activity(table 2).

Comparing control values with T-ALL values, significantly lower enzyme activities of APRT, PNP, 5'NT, ecto-5'NT and AMPD were observed in T-ALL lymphoid cells(table 2). On the other hand HGPRT and ADA activities were higher in T-ALL(table 2).

The activity of APRT, PNP, 5'NT, ecto-5'NT and AdKin were significantly lower in T-ALL cells as compared to nonBnonT-ALL cells(table 3). Only ADA activities were higher in T-ALL than in nonBnonT-ALL.

DISCUSSION

The reported data on ADA(5,12), PNP(2) and 5'NT(9) in acute lymphoblastic leukemia are confirmed in this study. ADA is clearly elevated in T-ALL, whereas in nonBnonT-ALL intermediate values between T-ALL and control enzyme activities are found. PNP is significantly decreased only in T-ALL. 5'NT activity assayed in lyophilized cells, or in intact cells

Table 3: Statistical Evaluation of Purine Enzyme Activities in Leukemic Cells from T-ALL vs. nonBnonT-ALL.

HGPRT	n.s.[1]
APRT	↓[2] ($p<0.05$)
ADA	↑↑[3] ($p<0.001$)
PNP	↓ ($p<0.02$)
5'NT	↓↓[4] ($p<0.001$)
ecto-5'NT	↓↓ ($p<0.001$)
AK	n.s.
AMPD	n.s.
AdKin	↓ ($p<0.02$)

[1]n.s. No significant difference. [2]↓Significantly decreased in T-ALL. [3]↑↑Highly significant increased in T-ALL. [4]↓↓Highly significant decreased in T-ALL.

as an ecto-enzyme, was hardly detectable in cells from T-ALL patients.

The elevated HGPRT activity and the decreased APRT activity in ALL have not been reported earlier according to our knowledge. The increased AdKin activity in nonBnonT-ALL is consistent with other data obtained by our group. It was found that the intracellular concentration of ATP was significantly elevated in cells from nonBnonT-ALL patients(6).

Data obtained in our laboratory and from others show that ADA, PNP and 5'NT activities fluctuate in several stages of lymphoid cell differentiation and maturation(3,4). Human thymocytes, immunologically characterized as young lymphoid cells, showed high ADA and low 5'NT activities. The ADA/PNP activity ratio in these cells was much higher than in peripheral blood lymphocytes(14). One might suspect that the differences in enzymatic activities in the subclasses of the leukemias mentioned reflect the stage of maturation in which the cells have been arrested, rather than an alteration caused by the leukemic transformation itself.

The most suitable enzymatic parameter to discriminate between normal lymphocytes on the one hand and leukemic lymphoblasts on the other hand seems to be the ADA activity. A clear cut discrimination between T-ALL and nonBnonT-ALL is provided by 5'NT(lower inT-ALL), and AdKin(also lower in T-ALL).

Due to these differences in enzymatic make-up one might speculate on employing these differences in the design of a more selective "enzyme directed chemotherapy". Theoretically, 6-mercaptopurine(6MP), a widely used anti-leukemic analogue of hypoxanthine, might be metabolized more effectively to its toxic nucleotide form by ALL cells than by normal lymphoid cells, since HGPRT activities are found to be lower in the latter(tables 1 and 2). Another illustration is the inhibition of ADA by analogues of adenosine, such as coformycin. Accumulation of nucleosides(e.g. deoxyadenosine) might be more toxic to T leukemic cells than to normal lymphocytes and nonBnonT leukemic cells. Phosphorylation of deoxyadenosine to deoxyAMP and susequently to deoxyATP can occur in non-leukemic and in leukemic cells to a comparable extent. However, since leukemic T cells hardly possess the capacity to dephosphorylate toxic deoxyribonucleotides(low 5'NT) this might lead to inhibition by

deoxyATP of ribonucleotide reductase and thus impairment of DNA synthesis(11).

The data presented in this paper suggest that enzymes of purine metabolism, -can be used as additional diagnostic tools in acute lymphoblastic leukemia,
-might be interesting as markers for lymphoid cell differentiation,
-might be suitable targets for enzyme directed chemotherapy.
Further studies to verify these statements are underway in our laboratory.

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chapter 7

PURINE METABOLISM IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA: BIOCHEMICAL MARKERS FOR DIAGNOSIS AND CHEMOTHERAPY

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ABSTRACT

Adenosine deaminase(ADA), purine nucleoside phosphorylase(PNP), 5'nucleotidase(5'NT), ecto-5'NT, hypoxanthine-guanine phosphoribosyltransferase(HGPRT), adenine phosphoribosyltransferase(APRT), adenosine kinase(AK), AMP deaminase(AMPD) and adenylate kinase(AdKin) activities were assayed in leukemic cells from bone marrow and/or peripheral blood of 43 newly diagnosed children with acute lymphoblastic leukemia(ALL). These enzyme activities have been investigated in relation to some other immunological markers. ADA activity was higher in E-rosette positive leukemia(E^+ ALL), while HGPRT, APRT, PNP, 5'NT, ecto-5'NT and AdKin activities were found to be lower in E^+ ALL as compared to E^- ALL. In common ALL(cALL) antigen positive leukemia, mean ADA activity was significantly lower as compared to $cALL^-$ leukemia, whereas PNP, 5'NT, ecto-5'NT and AdKin activities were significantly higher. cALL cells with cytoplasmic immunoglobulin M(IgM) heavy chains were found to have mean 5'NT activities twice as high as cALL cells lacking cytoplasmic IgM heavy chains. In two patients who had surface immunoglobulins(sIg) on their cell membranes, low 5'NT activities were found. When measuring enzyme activities after 2 to 4 days of prednisone monotherapy, only mean ADA and HGPRT activities decreased in nonBnonT-ALL. These decreases were not significant in T-ALL patients. Mean enzyme activities in the leukemic cells of five patients with relapse, were comparable to those in newly diagnosed patients, except for 5'NT, which was found to be within the activity range of control peripheral blood lymphocytes. It is concluded that ADA and AdKin activities are suitable as markers for E^+ ALL and $cALL^+$ leukemias, respectively. 5'NT might help to distinguish between cALL cells having and lacking preB characteristics. Since 5'NT activity may also be decreased in B-ALL, it is not suitable as a T-ALL marker. Enzymes of purine metabolism in leukemic relapse need further investigation.

Studies on patients with immunological dysfunctions have revealed that genetically determined deficiencies of certain enzymes of purine metabolism are intimately related to lymphocytic malfunction(14,15). This as a consequence of a differentiation and/or maturation block originating from the enzyme deficiencies(26). Since lymphoblastic leukemia may be cosidered as a disorder of lymphocyte differentiation (17), it is of interest to study systematically purine metabolism in lymphoid cells from patients with acute lymphoblastic leukemia(ALL). Such a study might serve three purposes:

1. The search for additional diagnostic biochemical markers in acute lymphoblastic leukemia.
2. The search for a relation between biochemical markers of lymphocytic differentiation and the immunological markers.
3. Based on the specific deviant enzymatic make-up of the malignant lymphoblasts, possibilities for specific chemotherapy might be explored.

In previously published studies, which focussed on a single or a few enzymes, inconsistent data have been reported on altered purine enzyme activities in ALL. Scholar and Calabresi(23) reported that activities of several enzymes of purine metabolism(various nucleotide kinases, adenosine deaminase and purine nucleoside phosphorylase) in lymphoid cells from two patients with ALL were not consistent. In one patient an adenosine deaminase(ADA) activity comparable with the control value was observed. In the other a seven-fold increased ADA activity was found. In ALL patients with a T cell phenotype(T-ALL), ADA activities were found to be 7-fold(25) or even 47-fold(9) higher than in normal lymphocytes. On the other hand, ADA levels in ALL patients without a T cell or a B cell phenotype(nonBnonT-ALL) were reported to be only two to four-fold elevated(25). Contrasting data were also reported to be higher in lymphoid cells from ALL patients than in peripheral blood lymphocytes(PBL) from controls, it should be mentioned, that in nonBnonT-ALL a higher ADA activity was observed than in T-ALL(24). Liso et al.(20) did not find a difference in ADA activity between nonBnonT-ALL and T-ALL.

Purine nucleoside phodphorylase(PNP) was reported to be decreased only in T-ALL(3). In T-ALL an inverse linear relationship has been reported between the 5'nucleotidase(5'NT) activity and the number of E-rosette positive cells(21). The mean activity of 5'NT in malignant T lymphoblasts was significantly lower as compared to normal lymphocytes and leukemic nonBnonT lymphoblasts.

In the present study we have systematically assayed nine different enzymes, namely: ADA(E.C. 3.5.4.4), PNP(E.C. 2.4.2.1), 5'NT(E.C. 3.1.3.5), ecto-5'NT, hypoxanthine-guanine phosphoribosyltransferase(HGPRT; E.C. 2.4.2.8), adenine phosphoribosyltransferase(APRT; E.C. 2.4.2.7), adenosine kinase(AK; E.C. 2.7.1.20), AMP deaminase(AMPD; E.C. 3.5.4.6) and adenylate kinase(AdKin; E.C. 2.7.4.3). These enzyme activities have been related with a number of clinical and immunological parameters in a series of 43 successive ALL patients.

We studied enzyme activities in relation to prednisone monotherapy in order to investigate the usefulness of enzyme activities as markers for the effectiveness of the chemotherapeutic regimen. From earlier studies by our group it was known that the mean percentage of cells in ($S+G_2+M$)-phase is lower after prednisone treatment(2).

MATERIALS AND METHODS

Patients

Fourty-three children with ALL(aged between 1 and 14 years) were investigated at the time of diagnosis. The study was made on bone marrow aspirates, except for the patients 38, 39, 57 and 67(table 1). The diagnosis was confirmed at the Reference Centre of the Dutch Working Group on Leukemia in Children(SNWLK), The Hague, The Netherlands. Out of this group, cells from 22 patients were investigated enzymologically again after 2 to 4 days prednisone monotherapy(dose 40 mg/m^2 once daily).

Isolation of Lymphoid Cells from Bone Marrow and/or Peripheral Blood

The lymphoid cells were isolated by the Ficoll-Isopaque gradient

centrifugation method as described previously(2), using tris buffered minimum essential medium(MEM) containing 5%(v/v) foetal bovine serum. These cells were used both for immunological and enzymological characterization.

Immunological Classification of ALL

The immunological classification was performed as a rule in almost completely homogeneous(>95%) leukemic cell populations from bone marrow and/or peripheral blood. Sheep erythrocyte(E) rosettes were used as a T cell marker(2). If more than 20% of the leukemic cell population formed E-rosettes at 4^0 C, the diagnosis T cell ALL was made. In a number of cases anti-T cell antiserum(Anti-Leu-1 monoclonal antibody, Beckton Dickinson, Sunnyvale, CA, USA) could be used in an indirect immunofluorescence assay, using fluorescein isothiocyanate(FITC)-labeled goat anti-mouse IgG antiserum. Again the 20% borderline was used as a criterion for positivity. As a criterion for B cell ALL the presence of more than 20% surface immunoglobulin(sIg) bearing cells was used. Immunofluorescence studies were performed using tetramethyl rhodamine isothiocyanate(TRITC)-labeled anti-human Ig(2). Leukemic cell populations that did not meet the criteria mentioned above were classified as nonBnonT-ALL.

Antisera against common-ALL antigen(cALL) could not be used in all cases studied. However, after anti-cALL antibody became available(J5 Coulter Clone monoclonal antibody, Coulter Electronics Inc., Hialeah, FL, USA), a number of patients of the nonBnonT-ALL group could be classified as cALL(more than 20% cALL positive cells) or unclassified-ALL(unc.-ALL; no significant numbers of membrane markers) according to Greaves' nomenclature(16). The binding of J5 to the leukemic cells was assessed by indirect immunofluorescence with fluoresceinated goat anti-mouse IgG antiserum.

PreB cell characterization was performed in an immunofluorescence assay on cytocentrifuge preparations of fixed bone marrow cells using TRITC-labeled anti-human IgM and FITC-labeled anti-human light chains (κ or λ) antisera. Cells lacking detectable sIg(determined as described above) but containing cytoplasmic IgM heavy chains in the absence of

light chains were denominated as preB cells(4).

Enzyme Assays

The activities of ADA, PNP, 5'NT, HGPRT, APRT, AK, AMPD and AdKin were assayed with radiochemical substrates, making use of our micromethods described previously(28). PNP was assayed using 8-^{14}C-hypoxanthine as a substrate. 5'NT was assayed on lyophilized cells, whereas ecto-5'NT activity was measured in intact cells, essentially according to Edwards et al.(11). With respect to AMPD and AdKin, the method of Leech and Newsholme(19) was adapted to our microsystem(28). Both bone marrow and peripheral blood samples were assayed from 16 patients, whereas in another 6 patients only peripheral blood samples were assayed(table 1).

Statistics

The data were statistically analyzed using the two-tailed Student's t-test with separate variance estimate.

Flow Cytophotmetric Studies

The number of bone marrow cells in ($S+G_2+M$)-phase was determined (Dept. of Hematology, University Hospital, Nijmegen, The Netherlands) using a flow cytophotometric technique described earlier(2).

In Vitro Effects of Prednisone and Prednisolone

To test the possible in vitro effects of prednisone and prednisolone, control peripheral blood lymphocytes were assayed for the enzymes as described onder "Enzyme Assays", in the presence of 10 ng/ml(0.03 μM) of one of the compounds.

Immunological Markers of the Patients

Several immunological characteristics determined in a group of 43 children with ALL are listed in table 1. In our group of patients, 29 cases could be classified as nonBnonT-ALL; subdivision in cALL and unc.-ALL was only possible after an anti-cALL antiserum became available to us(fig. 1). Eighteen nonBnonT-ALL patients(from the group of 29) could be investigated with the recently available anti-cALL serum. Sixteen cases were of the cALL type and 2 remained unclassified(fig. 1). From the 14 patients in our group, who didnot have a nonBnonT phenotype 12 were E-rosette or anti-T cell serum positive(>20% positive cells) and classified as T-ALL. Two patients in our group were of the B cell type(>20% sIg bearing cells).

Though in patients 6 and 7 most immunological markers could not be

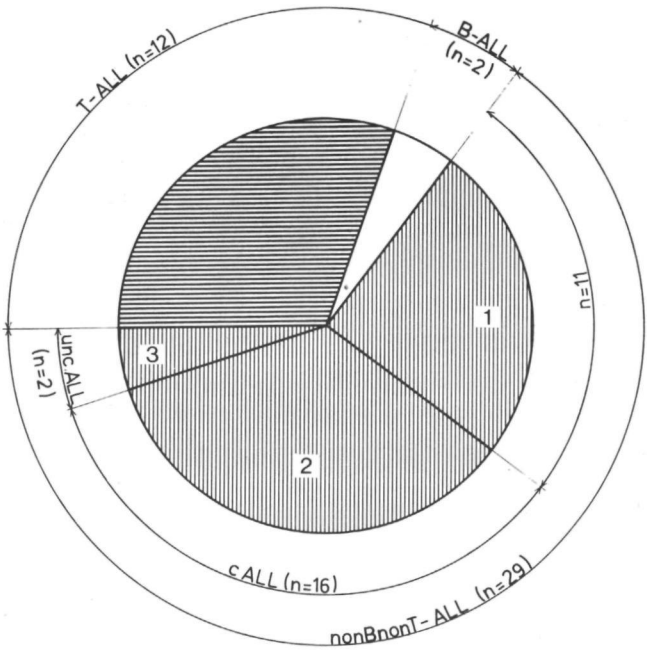

Figure 1: Immunological classification of the ALL patients studied.
1=nonBnonT-ALL patients; no further marker studies performed.
2=nonBnonT-ALL patients; cALL positive.
3=nonBnonT-ALL patients; unclassified ALL.

Table 1: Characteristics of Acute Lymphoblastic Leukemia Patients in this Study

Patient	Classification	WBC	%blasts	%E	%sIg	%cALL	%anti-T	BM/PB
1	nonBnonT	22.0	90	0	0	-	-	m
6	nonBnonT	15.8	100	-	-	-	-	m
7	nonBnonT	6.9	88	3	-	-	-	m
8	nonBnonT	4.0	80	4	0	-	-	m
10	nonBnonT	6.4	95	1	0	-	-	m
11	nonBnonT	0.8	90	3	0	-	-	m
12	unc.	232	95	3	0	0	0	m,b
13	nonBnonT	1.8	98	0	0	-	-	m
14	T	308	95	37	-	-	-	m
15	T	142	95	86	0	-	-	m,b
18	nonBnonT	7.2	95	2	0	-	-	m,b
21	nonBnonT	434	98	4	0	-	-	m
23	common	13.2	90	1	-	85	1	m,b
25	common	27.8	99	0	0	60	1	m,b
26	nonBnonT	12.9	65	0	0	-	-	m,b
29	nonBnonT	5.6	92	0	0	-	-	m,b
31	B	91.8	95	2	77	-	-	m,b
32	unc.	38.3	82	16	1	3	-	m,b
35	T	317	87	76	-	1	-	m,b
37	T	28.4	36	56	1	-	-	m
38	common	4.2	-	-	-	-	-	b
39	common	5.0	-	-	-	-	-	b
40	common	3.4	94	1	0	74	-	m,b
43	common	8.3	78	5	1	93	-	m,b
45	common	2.2	90	2	2	60	-	m
46	common	7.6	94	2	0	81	-	m
50	T	126	86	74	0	0	-	m,b
53	T	365	90	80	0	52	21	m,b
55	common	41.7	97	3	2	88	4	m
57	T	122	91	19	5	70	68	b
59	common	3.4	91	1	1	87	2	m
61	common	12.9	99	1	1	91	78	m,b
62	common	5.5	96	2	0	38	6	m
63	common	10.4	90	2	1	92	3	m
64	common	11.3	72	1	0	87	8	m,b
65	common	189	94	0	3	92	1	m
67	common	16.8	-	-	-	-	-	b
68	T	121	88	78	0	63	70	m
500	T	-	-	>20	-	-	-	m
501	T	-	-	>20	-	-	-	m
502	T	-	-	>20	-	-	-	m
503	T	-	-	>20	-	-	-	m
539	B	-	-	-	>20	-	-	b

The patients are numbered according to our computerized datafile. Missing numbers represent patients with other hematological malignancies. Initial white blood cell counts(WBC) are given in 10^9 cells/liter. The percentage of lymphoblasts is determined microscopically, using bone marrow smears stained with May-Grünwald Giemsa technique. Patients 38, 39, 57, and 67 were classified on the basis of peripheral blood findings.

determined, these patients were classified as nonBnonT-ALL on the basis of morphological aspects and a stimulatory capacity in the mixed lymphocyte reaction. In the peripheral blood of patients 38 and 39 more than 20% E^+ cells were found. The percentage of $cALL^+$ cells were 25 and 54, respectively. These patients were diagnosed as cALL, because the percentage of malignant cells in the peripheral blood were 29 and 54, respectively. The E^+ cells most probably represent the normal peripheral T cells. In the bone marrow of these patients no elevated numbers of E^+ cells were found. In patient 61, besides a high percentage of $cALL^+$ cells(91%) also many anti-T positive cells(78%) were found. This patient could be classified as belonging to a subgroup expressing both the cALL and T-ALL antigens(a "minor T-ALL" subtype according to Greaves' classification(16), or a "c/T-ALL subtype according to the classification of Huhn et al.(18)). By taking into account clinical parameters, as white blood cell count and mediastinal mass, we preferred to include the patient in the cALL group. Clinical parameters were also decisive for the diagnosis T-ALL in patients 53, 57 and 68, who showed amounts of $cALL^+$ and anti-T positive cells comparable to those found in patient 61.

Enzyme Activities and the E-rosette Marker

A relation between enzyme activities of purine metabolizing enzymes and subgroups of ALL according to the Greaves' classification(16) have been described by us before(29). In the present investigation we have looked for relationships with immunological markers in those groups of ALL patients having different enzyme activities. When plotting the enzyme activities measured, against the E-rosette marker, two groups of enzyme activities could be distinguished. With respect to ADA a group of patients with low to intermediate values(fig. 2) and relatively few

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The immunological markers E(rosette forming cells with sheep erythrocytes), sIg(surface immunoglobulin bearing cells), cALL(cells positive for the common-ALL antiserum) and anti-T(cells positive for the anti-T cell serum) are given as the percentage of positive cells. The cells used for enzymological characterization were isolated from bone marrow (m) and/or peripheral blood(b). Samples of the patients numbered 500-539 were kindly provided to us by colleagues from other clinics in the Netherlands. Detailed immunological phenotypes were not available to us.

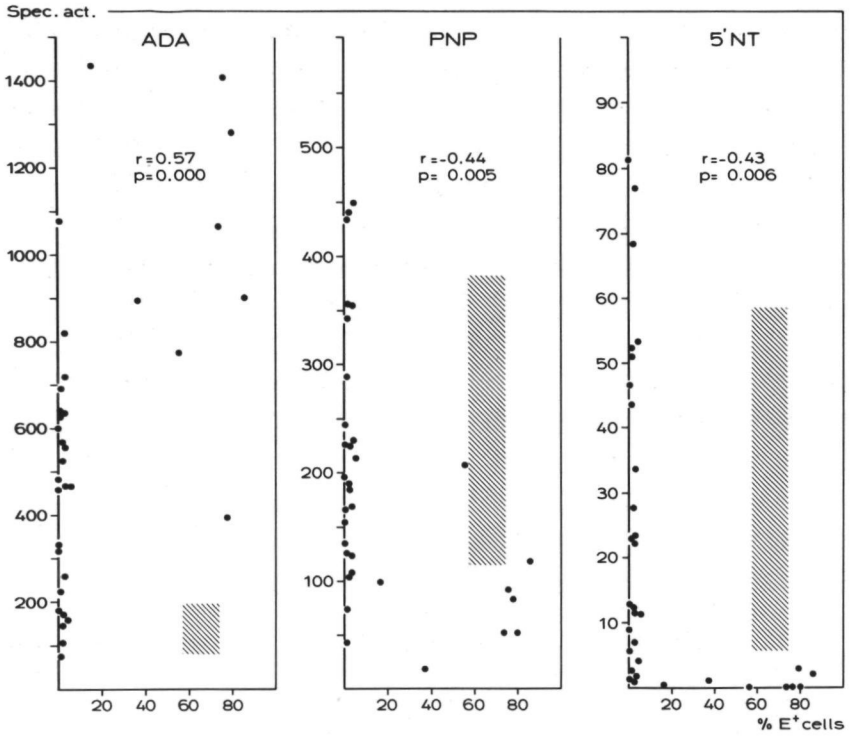

Figure 2: ADA, PNP, and 5'NT activity(10^{-9} moles/10^{6} cells.hour) in relation to the percentage of cells forming rosettes with sheep erythrocytes(E) in ALL. The grey area represents the range of these parameters in control peripheral blood lymphocytes. Correlation coëfficients (r) and the significances(p value)of these correlations are given.

E-rosetting cells and a group with high ADA activities and relatively many E-rosetting cells could be seen. In a similar way, distinctions could also be made in the case of low versus high activities of PNP, 5'NT(fig. 2), ecto-5'NT and AdKin. In order to perform statistics, a distinction had to be made between E^- and E^+ leukemias. Quite arbitrarily, but considering that this figure was already used in the immunological classification, the borderline between E^- and E^+ ALL's was considered to be at 20% E-rosette positive cells. It should be noted that shifting this border-line up or down 10% did not have any effect on the p values noted in table 2; only minor effects on the mean enzyme activities could be seen.

Table 2: Purine Enzyme Activities in Leukemic Bone Marrow Cells: the E-rosette marker

Enzyme	E-Rosetting Cells		p value
	<20%(n=26)	>20%(n=11)	
HGPRT	15.4 ± 7.0	10.8 ± 5.6	<0.05
APRT	10.8 ± 7.2	6.7 ± 2.8	<0.05
ADA	491 ± 314	1088 ± 452	<0.001
PNP	218 ± 116	115 ± 71	<0.005
5'NT	26.3 ± 25.0	1.3 ± 1.9	<0.001
ecto-5'NT	8.1 ± 7.3	0.7 ± 0.5	<0.001
AK	6.3 ± 4.1	6.3 ± 2.7	n.s.
AMPD	290 ± 315	157 ± 124	n.s.
AdKin	973 ± 531	220 ± 143	<0.001

Enzyme Activities are expressed in 10^{-9} moles/10^{6} cells.hour(mean ± s.d.). n.s.=not significantly different.

Significant differences in specific activities of purine enzymes were observed(table 2, fig. 2) from E^+ ALL(n=11) and from E^- ALL(n=26). Mean ADA activity was about 2.5 times higher in bone marrow cells from E^+ ALL as compared to E^- ALL($p<0.001$). On the other hand the mean PNP activity in E^- ALL was about twice the value of that in E^+ ALL($p<0.005$). Although considerable variation in 5'NT activities in ALL was observed, significantly lower and nearly undetectable 5'NT activities in bone marrow cells from E^+ ALL were evident. AdKin activity was significantly higher in E^- ALL than in E^+ blasts($p<0.001$).

In part of the patients(n=16), the above mentioned enzyme activities were also assayed in lymphoid cells from peripheral blood(data not shown). Statistical analysis of these data revealed that the same differences were found as with bone marrow cells, although the p values were less significant.

In fig. 2. it can be seen that there is a small overlap of ADA activity in the E^+ and E^- ALL cases. One E^+ ALL(patient 68) is in the ADA activity range(mean ± s.d.) of the E^- ALL and three E^- ALL's (patients 11, 12 and 40) are in the ADA activity range(mean ± s.d.) of the E^+ ALL. Though the mean PNP activities are significantly different, the overlap in enzyme activity of the E^- and E^+ ALL is considerable. ALL E^+ cases, except one(patient 68) are, in the lower part of the

E^- ALL activity range. 5'NT is low in all cases of E^+ ALL(fig. 2). Four patients(patients 12, 25, 31 and 32) from the E^- ALL group showed 5'NT activities in the E^+ ALL range. The difference between the two distinct populations was highly significant($p<0.001$, table 2). With the membrane-bound enzyme ecto-5'nucleotidase, essentially the same data were obtained($p<0.001$).

Enzyme Activities and the cALL Marker

There was a marked difference(fig. 3, table 3) in ADA activity between $cALL^+$(>20% cALL antiserum positive cells; n=15) and $cALL^-$ bone marrow cells(<20% cALL antiserum positive cells; n=4). ADA activity was higher in $cALL^-$ blasts($p<0.01$). On the other hand PNP, 5'NT and AdKin

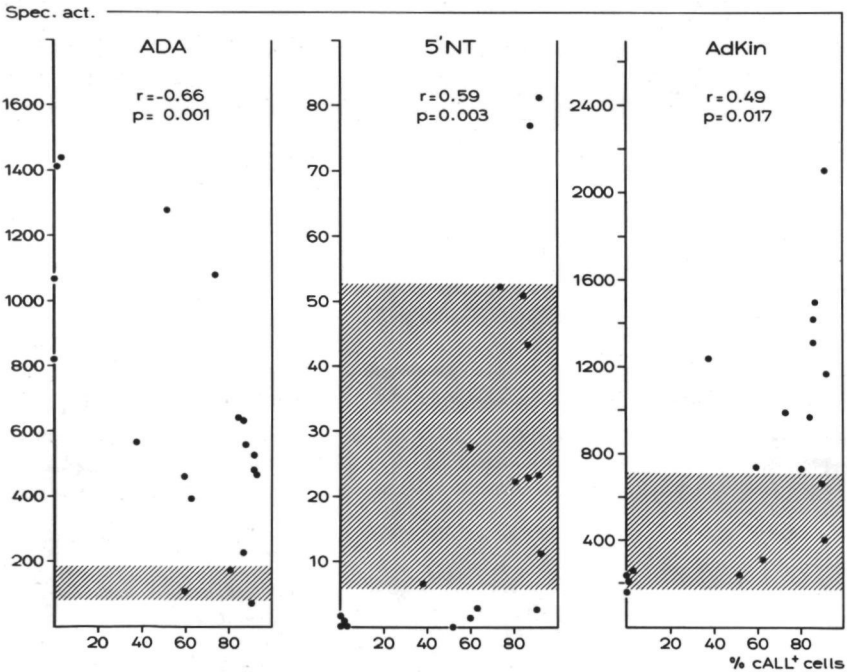

Figure 3: ADA, 5'NT and AdKin activity(10^{-9} moles/10^6 cells.hour) in relation to the percentage of cells positive for the cALL antiserum in ALL. The grey area represents the range of the respective enzyme activities in control peripheral blood lymphocytes. Correlation coëfficients (r) and the significances(p-value) of these correlations are given.

Table 3: Purine Enzyme Activities in Leukemic Bone Marrow Cells: the Common ALL Marker

Enzyme	cALL Positive Cells		p value
	<20%(n=4)	>20%(n=15)	
HGPRT	10.8 ± 1.6	14.5 ± 8.4	n.s.
APRT	7.8 ± 2.0	9.8 ± 7.4	n.s.
ADA	1186 ± 296	511 ± 330	<0.01
PNP	88 ± 24	197 ± 114	<0.005
5'NT	0.6 ± 0.8	28.5 ± 26.9	<0.001
ecto-5'NT	0.7 ± 0.6	8.8 ± 6.9	<0.001
AK	5.2 ± 1.2	5.1 ± 2.9	n.s.
AMPD	116 ± 70	251 ± 329	n.s.
AdKin	220 ± 41	982 ± 517	<0.001

Enzyme activities are expressed in 10^{-9} moles/10^6 cells.hour(mean ± s.d.). n.s.=not significantly different.

activities were higher(table 3) in bone marrow cells from $cALL^+$ leukemia ($p<0.005$, $p<0.001$ and $p<0,001$, respectively). Again these differences in enzymatic activities were less pronounced in cells derived from peripheral blood(data not shown).

From fig.3 it becomes clear that two patients(patients 40 and 53) out of the $cALL^+$ group have an ADA activity that is in the range of the $cALL^-$ patients. Concerning the 5'NT activity, two $cALL^+$ patients(patients 25 and 53) are in the $cALL^-$ activity range. In the case of AdKin this overlap is seen in one patient(patient 53).

Enzyme Activities and the Cytoplasmic IgM Heavy Chain Marker

Recently we have had the opportunity to test leukemic cells for the preB cell phenotype as defined by the presence of cytoplasmic IgM heavy chains(4). We tested 7 of our patients, who were classified as cALL. In the 3 patients who had cytplasmic IgM heavy chains present in their leukemic cells(patients 55, 65 and 67), the mean 5'NT activity was found to be 78.1 ± 2.7 10^{-9}moles/10^6 cells.hour. On the other hand the patients who did not show this preB marker(patients 40, 62, 63 and 64), had a mean 5'NT activity of 31.5 ± 20.3 10^{-9} moles/10^6 cells.hour ($p<0.02$). A comparable difference was observed with ecto-5'NT($p<0.05$).

HGPRT activity was higher in the cases in which no cytoplasmic IgM heavy chains were seen(21.5 ± 4.2 10^{-9} moles/10^6 cells.hour), compared to cytoplasmic IgM heavy chains containing cells(8.6 ± 1.5 10^{-9} moles/10^6 cells.hour). The p value was found to be below 0.005. All other enzymes tested showed comparable activities in these two groups of patients.

Enzyme Activities and the sIg Marker

Two patients(patients 31 and 539) appeared to have more than 20% sIg^+ cells. ADA activities(144 and 88 10^{-9} moles/10^6 cells.hour, respectively) were found to be lower than ADA activities found in $cALL^+$ leukemia(table 3) and E^+ ALL(table 2). PNP activities were found to be variable(440 and 135 10^{-9} moles/10^6 cells.hour, respectively). A very low activity was seen for 5'NT(0.9 and 0.1) and ecto-5'NT(0.2 and 0.1 10^{-9} moles/10^6 cells.hour, respectively), which was comparable to activities found in E^+ ALL. AdKin activities were in the range of E^+ ALL.

Enzyme Activities after Prednisone Monotherapy

In 22 of the ALL patients the systematic analysis of purine interconversion enzymes was repeated after 2 to 4 days of monotherapy with prednisone(dose: 40 mg/m^2 once daily in the morning). Bone marrow samples were obtained in the morning about 24 hours after the last dose of prednisone. The mean activities of HGPRT, APRT, ADA, PNP, AK, AMPD and AdKin in nonBnonT-ALL seemed to be decreased after prdenisone therapy (table 4). However, these differences were only statistically significant in the case of ADA($p<0.01$) and HGPRT($p<0.05$). In fig. 4 it can be seen that, with the exception of three cases, ADA activities decrease after prednisone therapy. Such a pattern was not seen with other enzymes. Prednisone and prednisolone were also tested in vitro for inhibition or stimulation of the various enzymes. Both compounds did neither inhibit nor stimulate the enzyme acticities determined.

Table 4. Purine Enzyme Activities in Leukemic Bone Marrow Cells. Effect of Prednisone Monotherapy

Enzyme	nonBnonT-ALL(n=17)		p value
	before monotherapy	after monotherapy	
HGPRT	15.4 ± 7.9	11.9 ± 6.0	<0.05
APRT	10.6 ± 6.6	7.4 ± 2.8	n.s.
ADA	550 ± 327	373 ± 177	<0.01
PNP	201 ± 117	196 ± 109	n.s.
5'NT	36.8 ± 26.3	39.5 ± 25.3	n.s.
ecto-5'NT	10.0 ± 8.1	12.1 ± 13.7	n.s.
AK	5.6 ± 3.8	4.9 ± 2.8	n.s.
AMPD	368 ± 379	327 ± 409	n.s.
AdKin	949 ± 497	840 ± 410	n.s.

Specific enzyme activities(10^{-9} moles/10^6 cells.hour) in bone marrow lymphoid cells of patients with nonBnonT-ALL before and after monotherapy with prednisone. n.s.=not significantly different.

Enzyme Activities and Leukemic Relapse

Leukemic cells from 5 nonBnonT-ALL patients(not included in table 1) have been enzymatically analyzed during relapse. Comparison of enzyme activities showed that in 26 newly diagnosed nonBnonT-ALL patients mean 5'NT activity was 27.6 ± 24.5 10^{-9} moles/10^6 cells.hour. In the 5 patients who were in relapse, mean 5'NT activity was found to be 10.0 ± 8.5 10^{-9} moles/10^6 cells.hour. The calculated p value was <0.01 which was considered to be statistically significant different. Other enzyme activities showed no statistically significant differences between newly diagnosed patients and patients in relapse.

DISCUSSION

Correlations between enzymological membrane markers in ALL and activities of enzymes of purine metabolism have been described by several authors(3,9,21,23,25,29). Our present findings confirm and extend these earlier reports. ADA was found to have the highest activities in E^+ ALL(1088 ± 452 10^{-9} moles/10^6 cells.hour, table 2), when comparing with E^- ALL(491 ± 314 10^{-9} moles/10^6 cells.hour, table 2) and normal

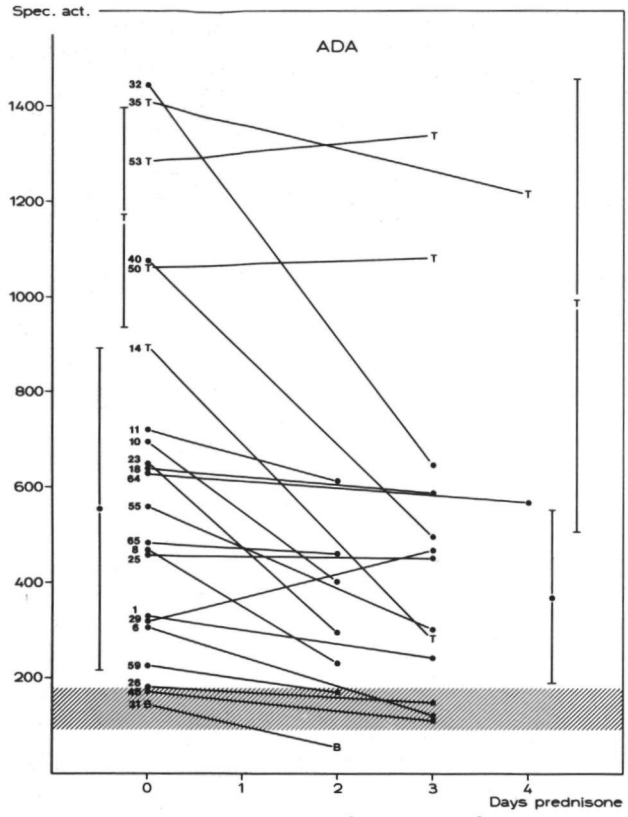

Figure 4: ADA activities(10^{-9} moles/10^6 cells.hour) in bone marrow cells from ALL patients, before and after 2 to 4 days monotherapy with prednisone. The vertical bars indicate the mean ± s.d. in nonBnonT-ALL(●; cALL and unc.-ALL included) and T-ALL(T). The grey area represents the range of ADA activities in control peripheral blood lymphocytes. The numbers refer to the patients in table 1.

control peripheral blood lymphocytes(134 ± 46 10^{-9} moles/10^6 cells.hour, ref. 29). In $cALL^+$ lymphoblasts the ADA activity was lower than in $cALL^-$ leukemia and comparable with that of E^- ALL(tables 2 and 3). The ADA activity of E^- ALL and $cALL^+$ leukemias is intermediary between the enzyme activity of E^+ ALL and control lymphocytes. The relative differences in ADA activity between control lymphocytes and lymphoblasts from cALL patients was in the same range as described by others(9,25). The patients in the E^+ ALL group are mostly diagnosed as T-ALL; the E^- ALL group showed an almost complete overlap with the $cALL^+$ group, whereas a similar overlap was observed between the E^+ ALL and the $cALL^-$ group.

Coleman et al.(9) however, found an ADA activity in T-ALL patients that was about 30 times higher than in control lymphocytes. The ADA activity in our group of E^+ ALL patients is about 8 times higher than in control lymphocytes. In our enzyme assay lyophilized lymphoid cells were used. Coleman et al.(9) assayed ADA activity in cell extracts prepared from sonicated lymphoid cells. We have previously shown that in sonicated control lymphocytes ADA activity is considerably lower than in lyophilized cells(27). This might be a reason for the differences in absolute ADA activity in sonicated cells(58 10^{-9} moles/10^6 cells.hour, ref. 9) versus lyophilized cells(143 10^{-9} moles/10^6 cells.hour, ref. 29). Apparently ADA activity in T lymphoblasts is not markedly affected by this difference in the lyzing procedure, since comparable ADA activities were found by both groups(9,29).

The essentially different results of Liso et al.(20), who found no difference of ADA activity between nonBnonT-ALL and T-ALL, may be ascribed to the different age of their patients. We were dealing with childhood ALL(1 to 14 years), whereas Liso's patients were mostly adults(12 to 68 years). Extrapolation of the biochemical findings described in the present paper to adult ALL, therefore does not seem to be valid.

The relatively low ADA activity found by Simpkins et al.(24) in T-ALL as compared to nonBnonT-ALL might be explained by the different way of expressing(per mg protein) the enzyme activity. If a much higher protein content per cell in T-ALL is present, the specific activity expressed on a protein basis becomes relatively lower than when expressed on a per cell basis.

In our two cases with sIg^+ ALL the ADA activity seems to be in the normal range or slightly dereased, the latter finding being in agreement with Coleman et al.(9). Recently in one patient with B-ALL an elevated ADA activity, as compared to normal lymphocytes, nonBnonT and T leukemic cells, was reported(22). Interestingly, the blasts of the latter patient were morphologically classified as being of the L_2 subclass(FAB classification). Our patients and those described by Coleman et al.(9) were found to be of the L_3(Burkitt's type) subclass. These data seem to indicate that heterogeneity exists in malignant B lymphoblasts, and that

ADA activity might be of help in the diagnosis of the different subclasses. However, the number of B-ALL patients investigated should be extended before this hypothesis can be confirmed or denied.

PNP activity in E^+ ALL was significantly lower than in E^- lymphoblasts. In control lymphocytes the PNP activity was about twice(209 ± 70 10^{-9} moles/10^6 cells.hour, ref. 29) that of the activity in E^+ ALL. These data are in agreement with findings by others(3,22). However, in one sIg^+ ALL we found a PNP activity that seemed decreased and was in the E^+ ALL range. In the other sIg^+ ALL patient we noted a PNP activity about twice that of control lymphocytes(29). Reaman et al.(22) have reported a PNP activity in a B-ALL patient, which was in the range of the T leukemic cells. In our opinion these different data can hardly be due to the different assay methods used. They could be merely due to the heterogeneity of B lymphoblasts derived from the B-ALL patients in the different studies.

5'NT activity in E^+ ALL is very low. Hardly any AMP degrading activity was seen in these lymphoblasts. In our $cALL^+$ leukemias the mean 5'NT activity(28.5 ± 26.9 10^{-9} moles/10^6 cells.hour, table 3) was about twice that of normal lymphocytes(18.4 ± 11.7 10^{-9} moles/10^6 cells.hour, ref. 29). A possible explanation for the extremely large range in 5'NT activity(s.d. is about 100% of the mean) is the heterogeneity within this subgroup of ALL. In this context an interesting observation was made. In three $cALL^+$ patients with preB characteristics the activity of 5'NT was about twice as high as in the cells of four $cALL^+$ patients without cytoplasmic IgM heavy chains. A similar difference was found with regard to HGPRT, but not with the other enzymes. Our finding of a higher mean 5'NT activity in $cALL^+$ leukemia's than in control lymphocytes is contradictory with the findings of Reaman et al.(21). Whether this difference is due to the different ways of correcting for phosphatase activity, which is not specific for the ribose-5'-P bond, is not clear. But it is known that such phosphatases can even be markers for certain subtypes of leukemia(6).

Our findings concerning ADA and PNP activities in sIg^+ ALL are partly in contradiction to the findings of others. However, the findings on the 5'NT activity in this subgroup of ALL seem rather unanimous. Like

other authors(9,22), we observe in both cases of B-ALL a decreased 5'NT activity that is in the range of the E^+ ALL. In conclusion, we cannot agree with the suggestion that 5'NT activity is an enzymological marker for distinct subclasses of ALL.

To our knowledge the differences in HGPRT and APRT activity in E^+ ALL and E^- ALL have not been described earlier. These differences were found to be not significant when comparing $cALL^+$ and $cALL^-$ leukemias. However, the numbers of patients were rather limited. Further exploration of the possible diagnostic value of these findings is indicated.

The activity of AdKin is significantly higher in E^- and/or $cALL^+$ leukemias than it is in E^+ and/or $cALL^-$ leukemias. To our knowledge this has not been described before. The higher activity of this nucleotide metabolizing enzyme in E^- and/or $cALL^+$ leukemia is in agreement with other data obtained by our group(10). It was shown that nonBnonT lymphoblasts had a significantly higher ATP concentration($1005 \pm 205\ 10^{-12}$ moles/10^6 cells) than control lymphocytes($500 \pm 150\ 10^{-12}$ moles/10^6 cells). In T lymphoblasts the ATP content is not significantly elevated. The possible implications of the elevated ATP concentration in certain cases of ALL for the energy charge of leukemic blasts are subject of further investigation.

Since it is assumed that lymphoblasts from patients with ALL are arrested in a certain stage of their differentiation(17) it is interesting to compare our biochemical findings in ALL with normal lymphoid cells from various differentiation stages. Lymphoid cells derived from the thymus are known to have relatively low 5'NT(12,30), high ADA (1,5,30) and low PNP(30) activities. In 6 separate thymocyte subfractions the ecto-5'NT activity ranged from 0.4 to 2.0 10^{-9} moles/10^6 cells.hour (30). Since lymphoblasts from T-ALL might have been arrested at different stages of the thymic differentiation, this can explain the relatively wide range of 5'NT activity in T-ALL. The same is the case with ADA and PNP activity. In relatively immature(phytohaemagglutinin unresponsive) thymocytes the ADA activity is about half the activity in relatively mature(phytohaemagglutinin responsive) thymocytes(30). PNP activity in the latter fractions is comparable with normal peripheral blood T lymphocytes, whereas in the immature fractions PNP activity is about 10% of

that value.

The biochemical consequences of the differences in purine enzyme activities for the leukemic cells have not been studied extensively yet. However, since the major differences found are in the ADA, PNP, 5'NT and AdKin activities, it might be anticipated that handling of purine nucleosides in E^+ ALL differs from that in cALL or in preB-cALL. Due to the relatively low 5'nucleotidase/kinase activity ratio, nucleosides (e.g. adenosine, deoxyadenosine, guanosine and deoxyguanosine) can be phosphorylated to a much higher extent in E^+ ALL than in other subtypes of ALL. Under certain conditions these phosphorylated nucleosides are toxic, especially to lymphoid cells(5,13). E^+ ALL cells e.g., might be more vulnerable to deoxyguanosine intoxication than other subclasses of leukemia. Such toxic effects were already found in thymocytes with a comparable enzymatic make-up(8). On the other hand the deaminating capacity(ADA activity) towards adenosine and deoxyadenosine in E^+ ALL cells is very high. Therefore adenosine and/or deoxyadenosine might be less toxic to these cells. Systematic analysis of purine nucleoside metabolism in leukemia might become helpful, not only in diagnosis but also for the development of a more selective chemotherapy in ALL.

The impact of the significantly decreased ADA and HGPRT activities in nonBnonT-ALL patients(table 4, fig.4) after prednisone monotherapy is not clear. No correlation appeared to exist neither with the ADA activity and the number of leukemic blasts in bone marrow, nor between the ADA activity and the number of cells in $(S+G_2+M)$-phase. Since nearly all patients showed a decreased ADA activity after prednisone therapy (fig. 4), further investigation seems to be justified.

It was surprising that when comparing newly diagnosed nonBnonT-ALL patients with patients in relapse, all purine enzyme activities were in the same range, except 5'NT. The finding that in nonBnonT-ALL relapse a lower 5'NT activity was observed might point to the development of a different leukemic (sub)clone. Before any final conclusions can be drawn more patients have to be studied. Longitudinal studies should be performed, measuring 5'NT and other purine enzymes, in order to establish whether such enzyme activities have also any prognostic significance in leukemic relapse.

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chapter 8

ENZYMOLOGICAL STUDIES IN CHRONIC LYMPHOCYTIC LEUKEMIA

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ABSTRACT

Adenosine deaminase(ADA), 5' nucleotidase(5'NT), ecto-5'NT, purine nucleoside phosphorylase(PNP), hypoxanthine-guanine phosphoribosyltransferase(HGPRT), adenine phosphoribosyltransferase(APRT), adenosine kinase (AK), AMP deaminase(AMPD) and adenylate kinase(AdKin) activities were assayed in peripheral blood lymphoid cells from 20 patients with B cell type chronic lymphocytic leukemia(CLL). Significantly decreased mean activities of ADA, 5'NT, ecto-5'NT, PNP and AMPD were observed when comparing B-CLL lymphoid cells with control peripheral blood lymphocytes (PBL). AK and AdKin) activities however, were found to be higher in B-CLL. Relatively wide ranges of ADA and 5'NT activity were observed. In patients with paraproteinaemia, 5'NT activity was found to be relatively high and in the range of the activities in normal PBL. ADA activity seemed to be slightly higher in patients without paraproteinaemia. No correlation could be found between the enzyme activities and the number of cells rosetting with sheep erythrocytes or bearing surface immunoglobulin(sIg). A relationship was suggested between 5'NT activity and Ig production.

INTRODUCTION

In the last decennium considerable differences in the activities of enzymes of purine metabolism have been described in various groups of human leukemias. In those papers data have been reported on a few enzymes in chronic lymphocytic leukemia(CLL): adenosine deaminase(ADA, 1,6,11,13,16), purine nucleoside phosphorylase(PNP, 1,2,13) and 5'nucleotidase(5'NT, 9,10). No simultaneous studies on several enzymes of purine metabolism in a group of CLL patients have been described. Mean ADA activity in leukemic cells from patients with CLL was reported to be 2 to 3-fold lower than in normal peripheral blood lymphocytes(PBL, 6,11). Simpkins et al.(14) however, recently described ADA activities in CLL lymphoid cells which were comparable to ADA activities in control PBL. In 6 out of 7 patients, Borgers et al.(2) found a weak histochemical staining for PNP in CLL lymphoblasts, in contrast to the clear staining of control PBL. In only one patient the histochemically determined PNP activity was comparable to the pattern observed in control lymphocytes. Ambrogi et al.(1) confirmed the decreased ADA and PNP activities in CLL. In their studies ADA activity was 2 to 3-fold lower and PNP activity was 3 to 6-fold lower as compared to PBL from normal donors. 5'NT activity was found to be low in lymphocyte membranes in 7 out of 10 CLL patients (9). In crude lymphocyte homogenates comparable results were obtained(9).

We have undertaken a systematic analysis of activities of nine enzymes involved in purine metabolism in lymphoid cells from 20 patients with B-CLL. ADA, PNP, 5'NT, ecto-5'NT, hypoxanthine-guanine phosphoribosyltransferase(HGPRT), adenine phosphoribosyltransferase(APRT), adenosine kinase(AK), AMP deaminase(AMPD) and adenylate kinase(AdKin) activities have been assayed in lymphoid cells from CLL patients.

MATERIALS AND METHODS

Patients

Eight female and twelve male patients, aging from 44 to 77 years, were investigated. Several immunological markers are listed in table 1. The percentage of cells with surface immunoglobulins(sIg) varied from

Table 1: Immunological Markers of Chronic Lymphocytic Leukemia Patients

Patient	Age	Sex[1]	Paraproteins[2]	E_s[3]	E_m[4]	sIg[5]
529	62	f	-	1	75	39
530	58	f	-	16	62	70
531	77	f	-	5	65	90
532	45	m	IgM/IgGκ	2	5	70
533	75	m	-	7	84	88
534	65	f	-	3	16	90
535	52	m	IgM/IgGλ	8	3	70
536	59	m	-	6	55	75
537	44	m	-	18	79	69
538	73	m	-	0	35	61
540	53	m	-	5	40	76
541	62	m	-	13	78	60
542	76	f	-	18	35	70
543	54	f	IgMλ	3	16	90
544	52	m	-	2	87	73
545	64	m	-	5	51	50
546	71	m	IgGκ	7	51	49
547	60	m	IgGκ	2	36	80
548	58	f	-	8	71	80
549	69	f	-	25	52	77

The patients are numbered according to our computerized data file. The missing number represents a patient with another hematological malignancy. [1]m=male, f=female. [2]Paraproteins present or paraproteins absent (-). [3]The percentage of cells forming rosettes with sheep erythrocytes. [4]The percentage of cells forming rosettes with mouse erythrocytes. [5]The percentage of cells bearing surface immunoglobulins.

39 to 90%; the percentage of cells rosetting with mouse erythrocytes(E_m) ranged from 13 to 87%. In the 4 patients with a relatively low amount of E_m^+ cells(<20%), the percentage of sIg^+ cells was 70 to 90%. Paraproteinaemia was observed in 5 patients, 3 of them had a low percentage of E_m^+ cells. In two cases a double paraproteinaemia was found(table 1).

Isolation of Lymphoid Cells

Cells were isolated from heparinized blood by Ficoll-Hypaque gradient centrifugation(d=1.077 gr/ml). T cells were identified by their capacity to spontaneously form rosettes with sheep red blood cells(E_s) and B cells were recognized by rosetting with mouse red blood cells(E_m), as described by Gupta et al.(5) and by the presence of sIg. The

percentage of sIg bearing cells was determined by immunofluorescence using affinity purified FITC-labelled antibodies as described by Gathings et al.(4).

Enzyme Assays

Enzyme activities were assayed making use of radiochemical substrates with an earlier described micromethod(17). HGPRT(E.C. 2.4.2.8) and APRT(E.C. 2.4.2.7) were assayed by measuring the conversion of hypoxanthine and adenine to their nucleotides, IMP and AMP respectively. PNP(E.C. 2.4.2.1) was assayed in both the catabolic and the anabolic direction. Since both methods essentially showed the same results, only the anabolic activity is given. The deamination of adenosine to inosine was measured as the ADA(E.C. 3.5.4.4) activity. 5'NT(E.C. 3.1.3.5) activity was determined in lyophilized cells as well as in intact cells (ecto-5'NT) with methods described by Ip and Dao(7) and by Edwards et al. (3), respectively. The two activities showed good correlations. Only 5'NT activity is given in tables 2 and 3. AK(E.C. 2.7.1.20) activity was assayed by following the conversion of adenosine to AMP. AMPD(E.C. 3.5.4.6) and AdKin(E.C. 2.7.4.3) were essentially assayed according to Leech and Newsholme(8) adapting their method to our micromethod. Enzyme activities are expressed in 10^{-9} moles product formed/10^6 cells.hour.

Table 2: Activities of Purine Enzymes in Peripheral Blood Lymphoid Cells from B-CLL Patients and from Normal Controls

Enzyme	Controls(n=20)	B-CLL(n=20)	[2]p value
HGPRT	6.9 ± 3.3[1]	7.0 ± 4.9	n.s.
APRT	13.6 ± 4.2	13.2 ± 6.5	n.s.
ADA	134 ± 46	49.2 ± 43.3	<0.001
PNP	209 ± 70	132 ± 96	<0.01
5'NT	18.4 ± 11.7	3.8 ± 6.8	<0.001
AK	7.9 ± 3.6	17.3 ± 6.7	<0.001
AMPD	267 ± 106	190 ± 105	<0.02
AdKin	265 ± 183	768 ± 425	<0.001

[1]Enzyme activities(mean ± s.d.) are expressed in 10^{-9} moles of product formed/10^6 cells.hour.
[2]p-values in the two tailed Student's t-test using the separate variance estimate
n.s.=not significantly different.

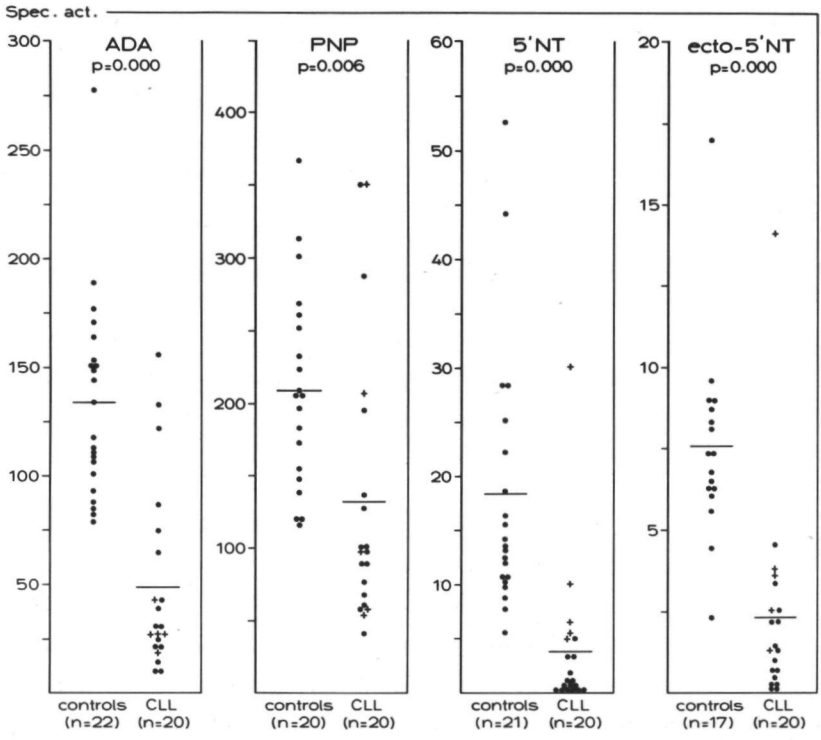

Figure 1: ADA, PNP, 5'NT and ecto-5'NT activities (10^{-9} moles/10^6 cells. hour) in control peripheral blood lymphocytes and lymphoid cells from patients with B-CLL. The mean values are indicated with a horizontal line. +=CLL patients with paraproteinaemia.

RESULTS

The mean activities obtained in the enzymatic analysis of a group of 20 B-CLL patients are depicted in figs. 1 and 2. Data on individual patients are given. Mean activities of ADA, PNP, 5'NT and ecto-5'NT in cells from CLL patients are significantly lower than in normal peripheral blood lymphocytes. Mean ADA activity is about $\frac{1}{3}$ of the control value, whereas PNP is only slightly lower(table 2). The most pronounced decrease in activity is found for 5'NT. In PBL from B-CLL patients the mean 5'NT activity is 3.8 10^{-9} moles/10^6 cells.hour(table 2). In contrast to the decreased enzyme activity levels described above, significantly higher values in B-CLL were observed for AK and AdKin. AK activity was about twice as high in B-CLL than in control PBL, whereas for AdKin a 3-fold

higher activity was found in B-CLL(table 2). Computerized statistical evaluation of the biochemical and immunological data was performed. No statistical differences or correlations in enzyme activities were detected in CLL patients in relation to the E_S or sIg markers. However, a correlation between the E_m marker and the 5'NT and ecto-5'NT activity seemed to exist. The correlation coëfficients were -0.57 and -0.50 for 5'NT and ecto-5'NT, respectively, with a statistical significance (p value) of 0.004 and 0.013, respectively.

When comparing CLL patients with and without paraproteinaemia, a significant difference was observed(table 3). The mean 5'NT activity was about 10-fold higher(table 3, fig. 1) in CLL patients with paraproteinaemia(range 5.1 to 30.1 10^{-9} moles/10^6 cells.hour) than in CLL patients without paraproteinaemia(range 0.1 to 3.4 10^{-9} moles/10^6 cells.hour).

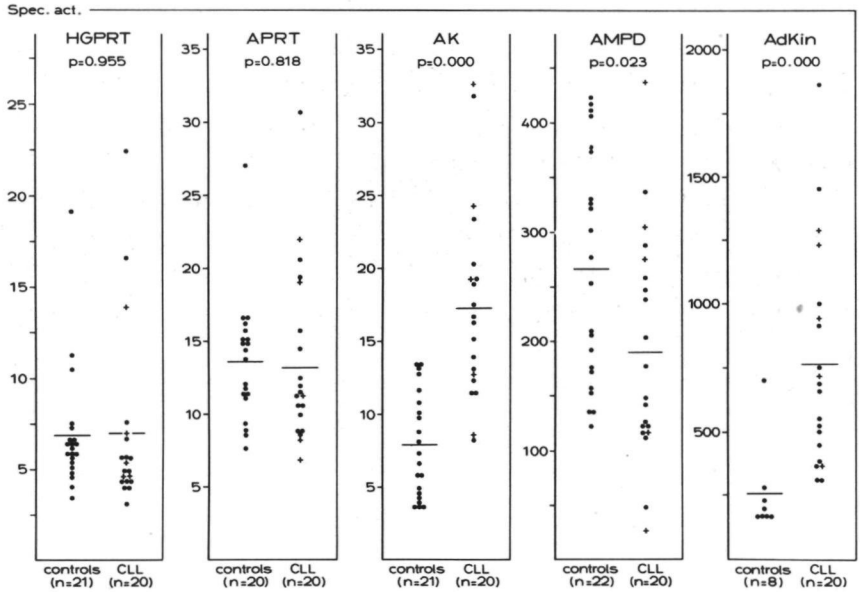

Figure 2: HGPRT, APRT, AK, AMPD and AdKin activities(10^{-9} moles/10^6 cells.hour) in control peripheral blood lymphocytes and lymphoid cells from patients with B-CLL. The mean values are indicated with a horizontal line. +=CLL patients with paraproteinaemia.

Table 3: Purine Enzyme Activities in B-CLL Patients with or without Paraproteinaemia

Enzyme	Patients WITH Paraproteinaemia (n=5)	Patients WITHOUT Paraproteinaemia (n=15)	p value[2]
HGPRT	7.1 ± 3.9[1]	6.9 ± 5.3	n.s.
APRT	13.5 ± 6.7	13.1 ± 6.7	n.s.
ADA	28.8 ± 8.7	56.0 ± 48.3	<0.05
PNP	154 ± 126	125 ± 88	n.s.
5'NT	11.5 ± 10.6	1.2 ± 1.5	<0.05
AK	19.5 ± 9.5	16.6 ± 5.8	n.s.
AMPD	232 ± 161	176 ± 81	n.s.
AdKin	915 ± 377	720 ± 441	n.s.

[1]Enzyme activities(mean ± s.d.) are expressed in 10^{-9} moles/10^6 cells. hour. [2]P values in the two tailed Student's t-test using the separate variance estimate. n.s.=not significantly different.

DISCUSSION

In contrast with findings in acute lymphoblastic leukemia(ALL, 18) mean ADA activity in B-CLL was a factor 3 lower than in normal PBL (table 2). This is in agreement with data reported by others(1,6,16). However, in contrast to the findings of Huang et al.(6) and Ambrogi et al.(1), who only observed ADA activities below the mean control level, we observed a considerable range from 8%(patients 533 and 537) to 116% of the mean control value(table 2). The ADA activity of four patients (patients 529, 531, 538 and 542) was within our control range(fig. 1). These conflicting results might be due to the fact that in the studies mentioned only limited numbers of patients were studied(8 and 4, respectively).

The correlation between ADA activity and the percentage of B cells in CLL patients that was described by Tung et al.(16), could not be confirmed in our group of patients. In the present study B cells were defined by the E_m and sIg markers. The complement receptor was used as a B cell marker in the study of Tung et al.(16). The fact that Simpkins et al.(14) reported comparable ADA activities in CLL lymphoid cells and in control lymphocytes, might be due to the difference in the enzyme assay. Simpkins et al.(14) used the indirect photometric assay of Tung et al.(16) in hypotonically lyzed cells. A selective lysis of cells

might be consequence of this method. Together with expressing the enzyme activity on a protein basis and not on a per cell basis, as we do, this might lead to different results. When comparing the ADA activities in CLL with various immunological markers, a significant difference was only found between CLL patients with paraproteinaemia and CLL patients without paraproteinaemia.

Although it is rather difficult to compare the histochemical findings of Borgers et al.(2) with our biochemical studies, they seem to be in agreement. The weak staining for PNP of the majority of lymphoid cells in 6 B-CLL patients most probably represents the relatively low PNP activity in most of the CLL patients. Like Borgers et al., we also find CLL patients, who show a PNP activity comparable to control values. As was also the case with ADA, Ambrogi et al.(1) observed a relatively narrow range of low PNP activity in B-CLL patients. In our group of CLL patients however, 7 patients had PNP activities within our control range (fig. 1). Again this discrepancy might be attributed to the relatively limited number of patients(n=7) studied.

Our findings of 5'NT activity in CLL are in agreement with data reported by others(10). In our group of patients a 5-fold lower mean 5'NT activity was observed than in control PBL. This difference would even be more pronounced when CLL B cells were compared with normal peripheral B cells, because these have a higher 5'NT activity than peripheral T cells(12,15,19). In our group, 4 out of the 20 patients showed 5'NT activities within our normal range(fig. 1). These patients (patients 532, 535, 543 and 546) all had paraproteinaemia(table 1). A significant correlation between the number of cells rosetting with mouse erythrocytes(E_m) and the 5'NT($r=-0.57$, $p=0.004$) and ecto-5'NT ($r=-0.50$, $p=0.013$) activities was found. When plotting these parameters, leukemic cell populations with relatively low 5'NT activity and a low percentage of E_m^+ cells as well as populations with low 5'NT activity and a high percentage of E_m^+ cells were seen. This apparent relation needs further investigations before conclusions can be drawn.

The differences in mean AK, AMPD and AdKin activities in CLL lymphoid cells as compared to PBL were found to be statistically significant(table 2). The ranges of AMPD in CLL patients and in control persons

were almost completely overlapping. Only the distribution of the individual activities in these ranges seemed to differ(fig. 2). The difference in mean AK and AdKin activities were more pronounced. Though 7 CLL patients fell in the normal range of the AK activity, none of the activities of the patients were below the mean control value(fig. 2). In the case of AdKin only one control value was found to be in the CLL activity range(fig. 2).

It should be noted that in general the activities of the catabolic enzymes 5'NT, ADA and PNP are lower in CLL patients than in control individuals. In contrast, the anabolic enzymes AK and AdKin show increased activities in CLL lymphocytes as compared to control PBL. Since AK and AdKin are involved in the synthesis and interconversion of nucleotides in cells, these enzymatic changes might have severe consequences for the energy charge in lymphoid cells from CLL patients. Analysis of intracellular concentrations of purine nucleotides has to be done to clarify this point.

Considering the mean 5'NT activity in B-CLL patients with and without paraproteinaemia, a significant difference was observed. The patients without paraproteinaemia showed a 10-fold lower mean 5'NT activity as compared with the patients with paraproteinaemia(table 3, fig. 1). A correlation between Ig production and 5'NT activity has been established earlier(3). It was shown that in patients with congenital agammaglobulinaemia 5'NT activities were decreased. In this disease the relatively low 5'NT activity might in part be attributed to the absence of peripheral B cells(12). Since B cells appear to have higher 5'NT activity than T cells(12,15,19), the consequence of this shift in lymphoid cell populations might be a lower net 5'NT activity. However, also in T cells of congenital agammaglobulinaemia a decreased 5'NT activity was observed(15). This supports the hypothesis that abnormal T cells may be responsible for the perpetuation of the disease and the low 5'NT activities.

Our findings may support a relationship between 5'NT activity and Ig production because B-CLL patients with a paraproteinaemia, which was related to the leukemic cell population, had a much higher 5'NT activity. However, the higher 5'NT activity may also be a reflection of a more

mature B cell population in the CLL patients with paraproteinaemia, irrespective of the capacity of Ig production. Moreover a relationship with the occurrence of IgM heavy chains in the cytoplasma has also been established in ALL cells, where preB leukemias(having cytoplasmic IgM heavy chains) had a twice as high 5'NT activity(18) as the nonBnonT-ALL patients(not having cytoplasmic IgM heavy chains). It should be emphasized however, that further studies have to be performed on the role of 5'NT activity in B and T lymphocyte differentiation and function.

ACKNOWLEDGEMENTS

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chapter 9

PURINE METABOLISM IN THYMOCYTE FRACTIONS

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ABSTRACT

Separation of human thymocytes according to size was performed using centrifugal elutriation. Six fractions were obtained. Two fractions (1 and 2) predominantly containing small sized thymocytes appeared to be poorly stimulated by phytohaemagglutinin(PHA). The number of cells agglutinating by peanut agglutinin(PNA) was relatively high in these fractions, whereas the number of cells staining for α-naphtyl acetate esterase(ANAE) was relatively low. It was concluded that fractions 1 and 2 mainly exist of immature thymocytes. The fractions predominantly containing medium sized cells(3 to 6), were considered to be relatively mature thymocytes. Fractions 3 to 6 were PHA responsive, contained a high number of ANAE staining cells and relatively few cells agglutinating by PNA. The activities of adenosine deaminase(ADA), purine nucleoside phosphorylase(PNP), ecto-5'nucleotidase(ecto-5'NT) and deoxycytidine kinase(dCK) were greatly different in these thymocyte fractions. Fractions 1 and 2 exhibited relatively low ADA, PNP and dCK activities, whereas these activities were higher in fractions 3 to 6. Ecto-5'NT activity was the highest in fraction 3. It could be shown that the ADA/PNP activity ratio is a marker for thymocyte maturation. Within thymocyte fractions a good correlation was found between the ecto-5'NT /dCK activity ratio and the inhibition of the PHA response by the nucleosides deoxyguanosine and deoxyadenosine. From these studies it was concluded that, since the number of cells contributing in the PHA response and the number of cells contributing in the purine enzyme activities differ considerably, it is not allowed to relate purine enzyme make-up with toxic effects of nucleosides in unseparated thymocytes.

The relevance of an intact purine metabolism for lymphoid cell function has been documented by a number of reports. Inherited deficiency of adenosine deaminase(ADA) is associated with a defect in both T and B cell development in a considerable number of patients with severe combined immunodeficiency disease(12,16). Purine nucleoside phosphorylase(PNP) deficiency is associated with selective T cell dysfunction (13,23). A relatively low 5'NT activity has been described(8) in patients with congenital X-linked agammaglobulinaemia. A metabolic consequence arising from ADA and PNP deficiency is the accumulation of deoxynucleosides in all somatic cells. Normally these compunds can be broken down to purine bases. Especially the lymphoid system has a high capacity to phosphorylate (deoxy)nucleosides to (deoxy)nucleotides(3,5,29). The latter compounds subsequently inhibit ribonucleotide reductase(10), an enzyme essential for DNA synthesis.

Not only with respect to lymphoid cell function, but also in relation to lymphocyte maturation, purine enzymes play an important roll. This is particularly true with regard to T cell maturation. The activities of deoxycytidine kinase and ecto-5'nucleotidase were found to be different in T cells at various stages of maturation. Thymocytes had a relatively low activity of ecto-5'nucleotidase(ecto-5'NT, 6) as compared to peripheral blood T lymphocytes(PBL-T). Deoxycytidine kinase (dCK) was higher in thymocytes than in PBL-T. The findings of relatively high ADA and low ecto-5'NT activities in thymocytes seem to parallel the findings of high ADA and low ecto-5'NT activities in T cell acute lymphoblastic leukemia(ALL, 18,22,27). The latter studies suggest that purine enzymes might be used as markers of T cells at various differentiation stages(4) and that the pathways of purine nucleoside handling can differ considerably during T cell differentiation. Thymocytes appear to possess a relatively high phosphorylating capacity for deoxynucleosides(6). Furthermore, due to a low ecto-5'NT activity(6,9) these cells have a low capacity to break down toxic nucleotides. Although evidence is accumulating that due to their enzymatic make-up, thymocytes are very vulnerable in inherited ADA and PNP deficiency, the exact mechanism of disturbed lymphocytic differentiation is not yet completely understood.

The different sensitivity towards deoxynucleosides of mature and immature T cells has been studied in vitro in relation to the proliferative response(6). However, these studies have been performed in unseparated T cells from peripheral blood and lymphoid organs(thymus, tonsil, etc.). Little is known about the intra-organ development and maturation of lymphoid cells. This applies particularly to the thymus: data on proliferative responses in which only a minority of cells is assayed(19) are related to enzyme activities in unseparated suspensions where all cells are assayed. We therefore studied purine metabolism at the intra-thymus level using fractions enriched in mitogen responsive cells. Using centrifugal elutriation we were able to separate six subsets of thymocytes. The enzymatic make-up of the various thymocyte fractions were studied in relation to the inhibition of proliferative response by deoxynucleosides.

MATERIALS AND METHODS

Thymocytes and Thymocyte Separation

Human thymus tissue was obtained from children(aged 2 to 7 months), undergoing cardiac surgery for other purposes. All thymus tissue included in this study shared a normal architecture in histological examination (formalin fixed and paraffine embedded tissue, stained with hematotoxilin eosin). The tissue was minced in small pieces; the thymocytes were harvested in Hepes buffered RPMI 1640 and washed twice. Separation according to size was performed by centrifugal elutriation under sterile conditions at 4^0 C, using a Beckman J21C centrifuge equipped with a JE-6 elutriation rotor. This procedure has been extensively described elsewhere(11). By stepwise decreasing the rotor speed, 6 fractions were obtained: the percentage of cells recovered in each fraction as well as the composition of each fraction is given in table 1. The data on phytohaemagglutinin(PHA) mitogen stimulation(stimulation indexes) in the fractions are also given(table 1). Data on immunological phenotyping for peanut agglutinin(PNA) binding capacity, for terminal deoxynucleotidyl transferase(TdT) staining(markers for immature cortical thymocytes) and for α-naphtyl acetate esterase(ANAE) staining(a marker for more mature, mainly medullary thymocytes) are depicted in figure 1. The

Table 1: Some Characteristics of Thymocyte Fractions After Separation on Size

Fraction	Cell recovery (%)	Mono-[1] cytes (%)	Large[1] sized cells (%)	Medium[1] sized cells (%)	Small[1] sized cells (%)	PHA[2] SI
Unseparated thymocytes	100	2	4	24	70	27
1	50	0	0	7	93	3
2	30	0	0	15	85	4
3	8	0	1	52	47	26
4	4	1	5	57	37	45
5	3	2	10	54	34	22
6	1	9	20	55	16	6

[1]May Grünwald Giemsa staining. [2]Results of PHA response are given as stimulation indexes(SI, 5 x 10^5 cells/culture).

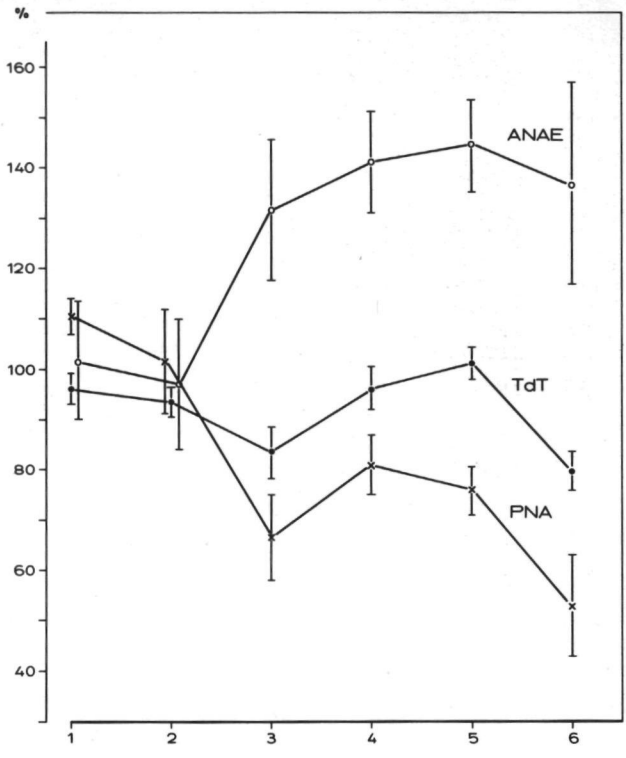

Figure 1: Relative shifts in percentages of PNA binding, TdT positive and ANAE positive cells in various fractions obtained by separation on size of human thymocytes. Results are given as mean percentages of positive cells(± s.d.), the initial values being set to 100%.

nature and characteristics of the present cell populations have been published in more detail elsewhere(19).

Peripheral Blood T Lymphocytes(PBL-T)

Heparinized blood was obtained from the donor of the thymus and from healthy control donors. Mononuclear cells were isolated by Ficoll Isopaque(d=1.077 gr/ml) density gradient centrifugation. T lymphocytes were purified by sheep erythrocyte rosetting, Ficoll-Isopaque density centrifugation and isotonic ammonium chloride lysis of erythrocytes, following well established procedures. The sheep erythrocytes were pretreated with 2-aminoethyl isothiouronium bromide hydrobromide(Sigma A-5879, ref. 15).

Mitogen Stimulation Tests

Cultures were performed in round-bottom microtiter plates(Greiner, Alphen, The Netherlands) in RPMI 1640 with bicarbonate, L-glutamin, antibiotics and 20% heat inactivated human AB serum. Cells were tested at 10^5 cells/culture. This amount of cells was chosen to enable comparison with PBL-T(usually cultured at 5×10^5 cells/culture). The rather low proliferative response after mitogen stimulation of unseparated thymocytes or of thymocyte subfractions was enhanced by supplementing the culture medium with both conditioned medium of human thymic epithelial monolayer cultures(17) and medium of human peripheral blood mononuclear cells after allogèneic and PHA stimulation. To obtain this latter conditioned medium, blood mononuclear cells of two donors were incubated together in the presence of PHA for two days, followed by extensive washing of the cells. After a second incubation for two days, the supernatant was harvested. Both conditioned media were used in the culture medium at a dilution of 1:30(optimal dilution assessed in prior experiments). The conditioned media showed no mitogenic effect in thymocytes nor blood mononuclear cells; the media had an enhancing effect on the proliferative response after PHA stimulation of unseparated thymocytes and of thymocyte subfractions, whereas no effect was observed on the response of peripheral blood mononuclear cells. The

culture medium, including the supplements, showed neglegible activity of purine enzymes. In mitogen stimulation 16 µg PHA(HA15, Wellcome, Beckenham, U.K.) was applied. In the study on (deoxy)nucleoside inhibition of the response, deoxyguanosine(Sigma D-900) was added in a final concentration of maximum 3 mM with a stepwise dilution(1:3) to a minimum of 1 µM. Deoxyadenosine(Sigma D-5875) was added in final molarities of maximal 300 µM and minimal 0.14 µM, in combination with deoxycoformycin (Warner-Lambert, Detroit, USA) in a molarity of 5 µM. In the inhibition studies with (deoxy)adenosine, cell cultures were first incubated with deoxycoformycin for 20 minutes to block all activity of adenosine deaminase present, followed by addition of (deoxy)adenosine. Cultures were incubated for about 90 hours at 37^0 C in humidified air with 5% CO_2. During the last 16 to 18 hours 1 µCi (methyl-^{3}H)-thymidine(5 Ci/mol, Radiochemical Centre, Amersham, U.K.) was present in each culture. The cells were harvested on glassfiber filters with an automatic harvester (Skatron, Lierbyen, Norway). Air dried filters were sampled in scintillation vials(Packard) and counted in toluene scintillator(Packard, containing 0.1 gr/l POPOP and 5 gr/l PPO). All cultures were performed in quadruplicate. Stimulation indexes(SI) were calculated as the mean counts per minute in mitogen stimulated cultures, divided by the mean counts per minute in unstimulated cultures. From (deoxy)nucleoside inhibition curves(counts per minute vs. concentration of inhibition) the concentration of nucleoside was determined, at which the proliferative response was 50% of the PHA response in absence of inhibitors.

Enzyme Assays

The activities of adenosine deaminase(ADA, E.C. 3.5.4.4) and purine nucleoside phosphorylase(PNP, E.C. 2.4.2.1) were assayed by making use of an earlier described micromethod(26), using radiochemically labeled adenosine and hypoxanthine, respectively. In 1,000 cells the ADA activity was determined by measuring the deamination of adenosine to inosine. Substrate and product were separated on PEI-cellulose thin layer chromatography plates. The specific activity was calculated from the amount of labeled inosine formed and expressed in 10^{-9} moles of inosine formed/10^6 cells.hour. Essentially the same method, with hypoxanthine as a substrate, was used to determine the PNP activity.

Ecto-5'nucleotidase(ecto-5'NT, E.C. 3.1.3.5) activity was assayed in intact cells using ^{14}C-AMP as a substrate. This method has been described earlier by Edwards et al.(8). Deoxycytidine kinase(dCK, E.C. 2.7.1.74) was assayed essentially according to Ullman et al.(25). In a 8 µl parafilm microcuvette(26) ½ µl of a cell suspension(12 x 10^6 cells/ml) was pipetted. The cells were frozen(15 min, -20° C) and lyophilized overnight. Three µl of an incubation mixture containing 5 µM ^{14}C-deoxycytidine (Radiochemical Centre, Amersham), 2 mM ATP, 5 mM $MgCl_2$, 0.5 mM cytidine, 0.5 mM deoxyuridine, 50 mM K_2HPO_4/KH_2PO_4(pH 7.4) and 0.33%(v/v) triton X-100(Packard Instrument, Brussels, Belgium) were added; incubation (1 hour, 37° C) was stopped in ice. One µl of the reaction mixture was spotted on PEI-cellulose thin layer chromatography plates(Machery-Nagel, SEL 300 PEI). The plates were prewashed in distilled water overnight. The chromatography was performed using 0.15 M NaCl as a solvent. Unlabelled deoxycytidine and deoxyCMP were cochromatographed and after detection under UV light the spots were cut out and counted in a liquid

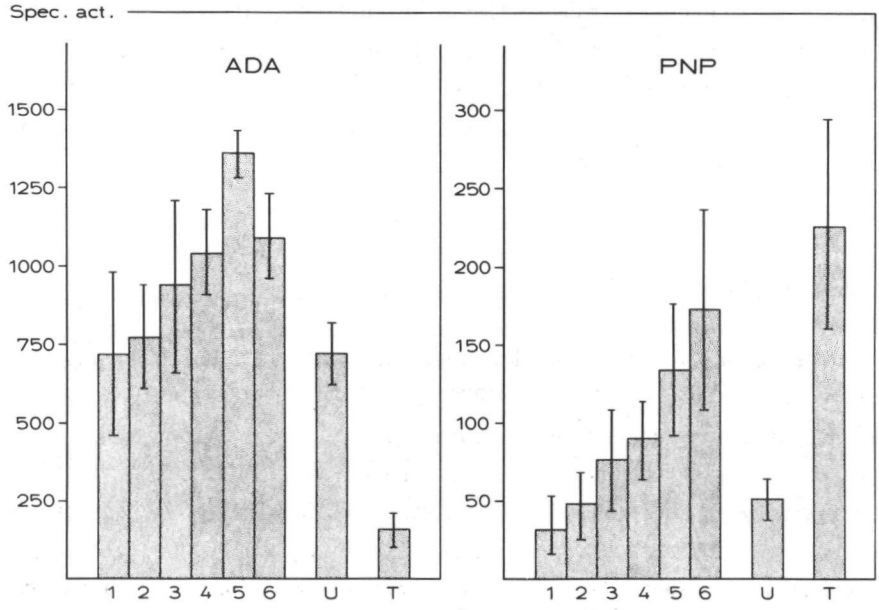

Figure 2: ADA and PNP activities(10^{-9} moles/10^6 cells.hour) in thymocyte fractions(1 to 6), unseparated thymocytes(U) and peripheral blood T lymphocytes(T). The mean values(± s.d.) of five separate experiments are indicated.

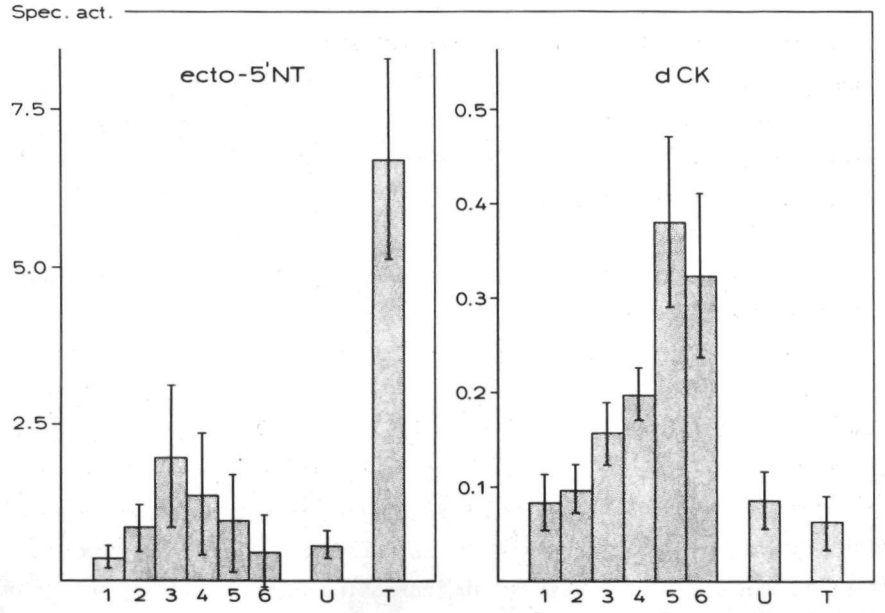

Figure 3: Ecto-5'NT and dCK activities(10^{-9} moles/10^6 cells.hour) in thymocyte fractions(1 to 6), unseparated thymocytes(U) and peripheral blood T lymphocytes(T). The mean values(± s.d.) of 5 separate experiments are indicated.

scintillation counter. Specific activity was expressed as 10^{-9} moles deoxycytidine nucleotides/10^6 cells.hour.

RESULTS

Enzyme Activities

The activities of ADA, PNP, ecto-5'NT and dCK were assayed in peripheral blood T lymphocytes(PBL-T), unseparated thymocytes(U) and thymocyte subfractions separated on size with the elutriation technique. The mean specific enzyme activities of 5 separate experiments are shown in the histograms of figs. 2 and 3. ADA activity was found to be relatively low in PBL-T(156 10^{-9} moles/10^6 cells.hour). In unseparated thymocytes ADA activity was considerably higher(721 10^{-9} moles/10^6 cells.hour). In the PHA unresponsive fractions 1 and 2, ADA activity was 722 and 772 10^{-9} moles/10^6 cells.hour, respectively. In the PHA responsive

fractions ADA activity ranged from 936 to 1357 10^{-9} moles/10^6 cells.hour (fractions 3 to 6). In contrast to the ADA activity PNP activity was highest(fig. 2) in the PBL-T(51 10^{-9} moles/10^6 cells.hour). In thymocyte subfractions the PNP activity increased with the fraction number and ranged from 35 10^{-9} moles/10^6 cells.hour in fraction 1 to 172 10^{-9} moles/10^6 cells.hour in fraction 6.

The activity of dCK(fig. 3) was found to be comparable in unseparated thymocytes and PBL-T(0.088 and 0.064 10^{-9} moles/10^6 cells.hour, respectively). As was the case with ADA and PNP the lowest dCK activities in thymocyte subfractions were found in the PHA unresponsive fractions 1 and 2(0.082 and 0.098 10^{-9} moles/10^6 cells.hour, respectively). In the PHA responsive fractions 3 to 6 the dCK activity ranged from 0.156 to 0.38 10^{-9} moles/10^6 cells.hour. The results of ecto-5'NT activity determinations were essentially different compared to the enzyme activities described above(fig. 3). Whereas an increasing enzyme activity was seen with the fraction number in the case of ADA, PNP and dCK, the maximal ecto-5'NT activity in thymocyte subfractions was observed in fraction 3 (2.0 10^{-9} moles/10^6 cells.hour). The activity in the remaining subfractions ranged from 0.4 10^{-9} moles/10^6 cells.hour in fraction 1 to 1.4 10^{-9} moles/10^6 cells.hour in fraction 4. Ecto-5'NT activity in PBL-T was about 12-fold higher than in unseparated thymocytes. It should be emphasized that, although the standard deviations especially from ecto-5'NT are rather high, the activity patterns in all five experiments are basically comparable; e.g. in every experiment maximal 5'NT activity was found in fraction 3.

Inhibition of PHA Response by (Deoxy)nucleosides

Deoxyadenosine and adenosine were added in different concentrations to PHA stimulated cultures after pre-incubation for 20 min. with 5 μM deoxycoformycin. Deoxyguanosine was used without any further additives. In fig. 4 the results of an experiment carried out in quadruplicate are shown. The results are presented as the (deoxy)nucleoside concentration at which the PHA response was 50% of the response without any addition. Four comparable experiments were carried out, which essentially showed the same results. The mean PHA responses without any addition of

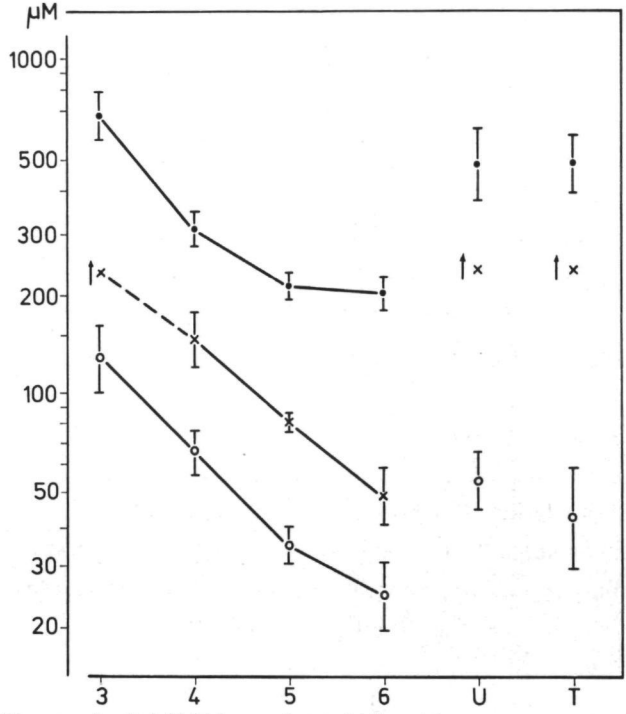

Figure 4: Inhibition of proliferative responses after PHA stimulation by adenosine(x), deoxyadenosine(o) and deoxyguanosine(●) in thymocyte fractions(3 to 6), unseparated thymocytes(U) and peripheral blood T lymphocytes(T). The (deoxy)nucleoside concentrations given, indicate the concentration at which 50% of the PHA response without any addition is seen(n=4).

nucleosides or deoxycoformycin in the experiments of fig. 4 are for PBL-T: 35,000 cpm; unseparated thymocytes(U): 7,000 cpm; fraction 3: 15,000 cpm; fraction 4: 50,000 cpm; fraction 5: 30,000 cpm; fraction 6: 40,000 cpm. Fractions 1 and 2 were considered to be unresponsive(<1500 cpm). When deoxyguanosine was added, the PHA responses of PBL-T and unseparated thymocytes were inhibited to a comparable extent. Thymocyte fractions 3, 4, 5 and 6 were inhibited each to an increasing extent by deoxyguanosine(fig. 4). Fractions 1 and 2 could not be evaluated because these fractions showed only marginal proliferative responses after PHA stimulation. Comparing the effects of deoxyadenosine and adenosine in the presence of the ADA inhibitor deoxycoformycin, cells were more sensitive to deoxyadenosine than they were to adenosine. Again PBL-T and unseparated thymocytes were inhibited by deoxyadenosine to a

comparable extent. In thymocyte subfractions this inhibition was minimal in fraction 3, whereas fraction 6 was mostly inhibited.

DISCUSSION

The application of a micromethod using 1,000 to 6,000 cells per incubation on one hand and an elutriation technique for the isolation of small subpopulations of thymocytes(11) on the other hand enabled us to study several aspects of purine metabolism in thymocyte subfractions.

The finding of relatively high ADA activity in thymocytes as compared to PBL-T is compatible with the high levels of ADA activity observed in T-ALL(4,22,27). Using an immunohistochemical technique, Chechik et al.(4) have elegantly shown that cells positive for a human thymus associated leukemia antigen, which appeared to be ADA, were predominantly present in the cortical regions of the thymus. In the thymic medulla, ADA positive cells were observed less. It has been shown that the thymic cortex contains less mature lymphocytes than the medulla(2). Apparently it is not correct to compare the qualitative results of Chechik et al. with the quantitative data we have obtained. In our experiments highest ADA activities are observed in the PHA responsive(i.e. more mature, medullary thymocyte containing) fractions. Fractions 3 to 5 contained relatively high numbers of ANAE positive cells and little PNA positive cells(fig. 1). This indicates that fractions 3 to 5 are relatively mature as compared to fractions 1 and 2.

In rat thymocytes fractionated on Ficoll-Hypaque density gradients the highest levels of ADA activity were observed in fractions enriched in immature cortical thymocytes(1). These results lead to the conclusion that intrathymic ADA activities in the rat are expressed differently from humans. In contrast to the findings on ADA, the findings on PNP activities in rat and human thymocyte subfractions seem to be similar. As was the case in our PHA unresponsive human thymocyte fractions, lowest PNP activities are also found in rat thymocyte fractions enriched for cortical thymocytes(i.e. less mature). Highest PNP activities are found in PHA responsive human thymocyte fractions(figs. 1 and 2) and in medullary rat thymocyte enriched fractions(1). Although there is an

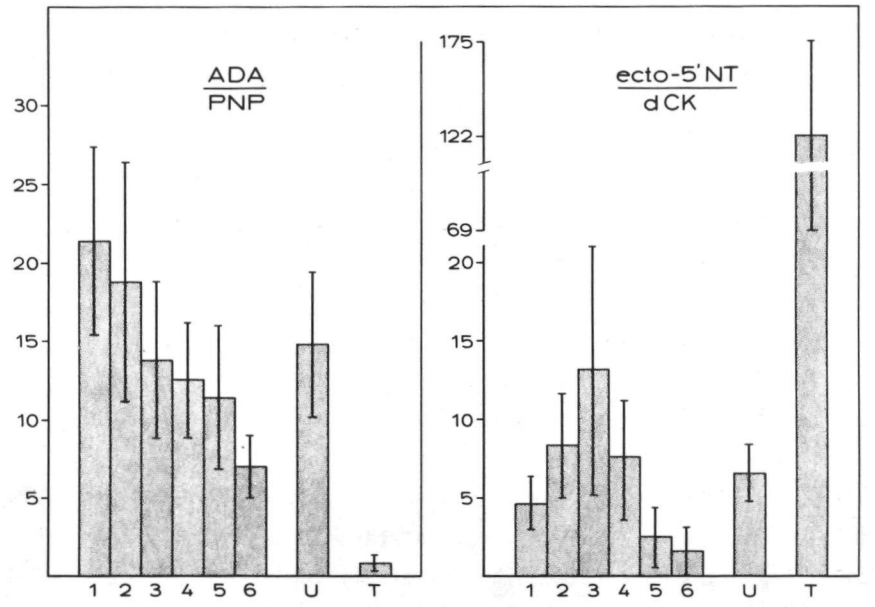

Figure 5: ADA/PNP and ecto-5'NT/dCK activity ratios in thymocyte fractions(1 to 6), unseparated thymocytes(U) and peripheral blood T lymphocytes(T). The mean values(± s.d.) of 5 separate experiments are indicated.

apparent contradiction regarding ADA activity between the results obtained in rats and in humans, it seems justified to state that stages in T cell maturation in the thymus can be characterized by either the absolute level of PNP or the relative levels of ADA and PNP(fig. 5). PHA unresponsive thymocytes have relatively high ADA/PNP activity ratios (21 and 19), whereas PHA responsive thymocytes have relatively low ADA/PNP activity ratios(14 to 7). The same applies to unseparated thymocytes and peripheral blood cells. The mean ADA/PNP ratio in thymocytes is found to be 15, in PBL-T this ratio is 1.

Although ADA and PNP appear to be of vital importance for normal lymphoid cell differentiation, little is known about the exact role of both enzymes in precursor T cells. Shore et al.(21) have shown that precursor T cells derived from ADA deficient patients may be induced to form rosettes with sheep red blood cells by incubation with normal thymic epithelial monlayers. When these monolayers are treated with an ADA inhibitor, this induction is inhibited. It is known that deoxyadenosine

in the presence of an ADA inhibitor blocks proliferative responses(28). However, from the fact that, when once entered blastogenesis thymocytes become less vulnerable to the addition of deoxyadenosine and deoxycoformycin(24), it might be concluded, that ADA activity is more vital for thymocytes in early maturation stages.

The ratio of deoxynucleoside phosphorylating capacity and deoxynucleotide dephosphorylating capacity has been subject of a number of studies. Since no substantial difference between deoxynucleotide forming capacities was observed between T and B lymphoblasts(29), another metabolic basis for the differential toxicity of deoxynucleosides was suspected(5,7). The higher levels of ecto-5'NT activity in B cells was found to provide B cells with a more effective way of breaking down deoxynucleotides(3). In order to predict the possible effects of nucleosides on T cells of various maturation stages, the activity ratio of ecto-5'NT/dCK was calculated. The relatively low ecto-5'NT activity in thymocytes as compared to PBL-T was described previously(6,9). The difference found between dCK activity in PBL-T and thymocytes(6) was not found in our studies. Comparable activities of dCK were observed in thymocytes and PBL-T(fig. 3). Whereas dCK activity was increasing with cell size, ecto-5'NT had a maximal activity in fraction 3. When the relationship between nucleoside toxicity and the nucleotide synthesis/nucleotide breakdown ratio also applies to the intrathymic situation, the conclusion seems justified that the cells in fraction 3 are most vulnerable to nucleoside toxicity.

Inhibition studies with nucleosides showed a comparable effect of deoxyguanosine and deoxyadenosine to PBL-T and unseparated thymocytes. Previously it was reported that thymocytes are more vulnerable towards deoxyguanosine intoxication than PBL-T(6). In the latter study 50% inhibition of the PHA response was observed at a deoxyguanosine concentration of 150 μM in PBL-T. This lower level of deoxyguanosine intoxication as compared to our studies might be explained by the different numbers of cells used. Cohen et al.(6) used 5×10^4 cells/well, whereas we used 10^5 cells/well. Yet, why the relative effects on PBL-T and thymocytes are found to be different, remains unclear to us. It might well be that the addition of the conditioned media in order to enhance proliferative responses accounts for this phenomenon. The activities of

PNP as well as ADA were assayed in these supplements and found to be neglegible. A change in deoxynucleoside toxicity induced by the conditioned media might be possible in the more immature cells. Deoxyguanosine and deoxyadenosine are believed to interfere with DNA synthesis through their phosphorylated derivatives deoxyGTP and deoxyATP, respectively(14,20). These nucleotides inhibit ribonucleotide reductase which catalyzes the formation of deoxyribonucleotides necessary for DNA synthesis(10). In the thymocyte subpopulations, which were all cultured under identical conditions, we find an excellent correlation between deoxyguanosine and deoxyadenosine effects on the PHA response and the enzymes that may influence the concentrations of deoxyGTP and deoxyATP. Relatively low ecto-5'NT activity may provide the cells with a rather poor possibility to breakdown formed nucleotides. Previously the reduced rate of deoxyATP degradation in T cell lines as compared to B cell lines was explained by a relatively low 5'NT activity(3,29). Nucleoside kinases, the enzymes that catalyze the phosphorylation of deoxyguanosine and deoxyadenosine to their respective nucleotides, are supposed to play an essential role in the specific intoxication of the lymphoid system in ADA and PNP deficiency(25). From our experiments in thymocyte subfractions it is indicated that the activity ratio of ecto-5'NT/dCK(fig. 5) is related to nucleoside toxicity in these subfractions. When the statistical correlation is calculated between the ecto-5'NT/dCK activity ratio of fractions 3 to 6 and the concentration of (deoxy)nucleosides at which 50% of the PHA response is seen, a highly significant correlation coëfficient(Pearson's correlation coëfficients >0.9) is found for the three nucleosides tested. In other words, the higher the ratio, the higher deoxynucleoside concentrations are needed to obtain 50% inhibition of the PHA response.

The knowledge of this biochemical heterogeneity with regard to enzymatic make-up and nucleoside toxicity in the intrathymic situation is of importance when studying mechanisms of cytotoxicity in relation to mitogenic responses. Since cells contributing to the PHA response (fractions 3 to 6) and cells hardly contributing to the PHA response (fractions 1 and 2) differ considerably in their relative and absolute enzyme activities(figs. 2, 3 and 5), and a relation is indicated between some of these enzyme activities and nucleoside intoxication, it is not allowed to relate enzyme activities and nucleoside induced

inhibition of the PHA response in unseparated thymocytes.

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chapter 10

DIFFERENTIAL IN VITRO EFFECTS OF ADENOSINE AND DEOXYADENOSINE ON HUMAN T AND B LYMPHOCYTE FUNCTION

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ABSTRACT

Human peripheral blood lymphocytes were stimulated with different mitogens(phytohaemagglutinin, PHA; pokeweed mitogen, PWM; concanavalin A, ConA; and protein A, SpA) known to activate different subpopulations ^{3}H-Thymidine and ^{3}H-leucine incorporation were studied in the presence and absence of adenosine, deoxyadenosine and the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)-adenine hydrochloride(EHNA). In addition the effects on in vitro IgG secretion induced by PWM or SpA have been studied.

Adenosine and deoxyadenosine added to the cultures together with EHNA inhibit the T cell mitogenic response. ^{3}H-Thymidine incorporation in ConA and PWM stimulated T cells is more affected by EHNA and (deoxy)-adenosine than in PHA stimulated cultures. At very low EHNA concentrations a facilitating effect on the protein secretion is observed in the PHA stimulated cultures. These findings suggest that different subsets of T cells have different sensitivities. EHNA and adenosine inhibit the IgG production by PWM stimulated cells. This inhibition might be secondary to a blockade of either the B cell or the T-helper cell activity, or to enhancement of T-suppressor activity. We favour the latter possibility. Deoxyadenosine in combination with EHNA enhances IgG secretion in PWM stimulated cells at low concentrations, whereas higher concentrations have an inhibitory effect.

Adenosine and deoxyadenosine are known inhibitors of the in vitro proliferative response of peripheral blood lymphocytes(PBL) to various mitogens(3,4,9,11,13,18,19). Erythro-9-(2-hydroxy-3-nonyl)-adenine hydrochloride(EHNA), an inhibitor of the enzyme adenosine deaminase(ADA), potentiates the inhibitory effects of both adenosine and deoxyadenosine. The incorporation of ^{3}H-leucine into protein appeared to be a more sensitive index of adenosine toxicity than the incorporation of ^{3}H-thymidine (3). A differential sensitivity of cultured human T and B lymphoblasts towards the toxic effects of adenosine and deoxyadenosine has been observed: T cells are more sensitive than B cells(5,15). On the basis of ^{3}H-thymidine incorporation, mouse splenic cells stimulated by lipopolysaccharide(LPS) were less sensitive to the addition of adenosine than the concanavalin A(ConA) stimulated cells(17). Using a plaque forming cell assay, a biphasic effect of adenosine was seen on the in vitro antibody production in mice. Concentrations of adenosine around 1mM stimulated antibody production in vitro, whereas concentrations above 1.5 mM had inhibitory effects(17).

The effects of adenosine and deoxyadenosine on subpopulations of regulatory lymphoid cells are poorly documented, both in animal models and in humans. In a first approach to this issue we have compared the effects on ^{3}H-thymidine and ^{3}H-leucine incorporation in parallel cultures stimulated with different mitogens known to activate different subpopulations(phytohaemagglutinin, PHA; ConA; pokeweed mitogen, PWM; protein A from Staphylococcus aureus, SpA). In addition, the effects on in vitro IgG secretion induced by PWM or SpA(both T dependent polyclonal B cell activators) have been studied.

Adenosine and deoxyadenosine probably exert their effects according to different mechanisms. These may include intra- and extracellular events. Intracellular accumulation of deoxyATP, decreased phosphoribosylpyrophosphate(PRPP) availability and inhibition of transmethylation reactions might occur(for review see 16). Ectracellular mechanisms might involve activation of adenosine receptors leading to increased or decreased levels of cyclic AMP within the cell depending on the type of adenosine receptor involved(2). It seems likely that different types of

cells involved in the immune response express different sensitivity to one of these mechanisms.

MATERIALS AND METHODS

Cell Preparation

PBL from healthy volunteers were prepared by centrifugation of heparinized blood, diluted with two volumes of Hank's balanced salt solution on Ficoll-Metrizoate(gravity 1.077 gr/ml; 1).

Mitogens

PHA(PHA-P, Wellcome) was used in a final concentration of 1 μgr/ml; ConA and PWM were purchased from Gibco and used at a final dilution of 5 μgr/ml and 1/100(v/v), respectively. Purified protein A was obtained from Pharmacia Fine Chemicals AB(Uppsala, Sweden, batch No. C12414); it was used in a final concentration of 10 μgr/ml.

Culture Conditions

Cells(25×10^4) were cultured in flat bottomed microplates(Microtest II, No. 3040F; Falcon, B.D. Oxnard CA) in 250 μl culture medium. This consisted of Hepes buffered RPMI 1640(Flow Labs., Rockville, MD) supplemented with 40 mM glutamine, 40 μgr/ml gentamycin, 0.05 μM 2-mercaptoethanol and 10%(v/v) foetal calf serum(FCS; Microbiological Ass., Bethesda, MD, batch No. 90874). All cultures were set up in triplicate. Incubation was performed at 37^0 C in a humidified atmosphere with or without 5% CO_2.

Measurement of DNA Synthesis

Five microCuries of methyl-^{3}H-thymidine(specific activity 10 Ci/mmol, IRE., Fleurus, Belgium) in 0.05 ml RPMI was added to the culture

18 hours prior to harvesting. Cells were collected on glass fiber filter paper(Reeve Angel fibre filter, grade: 934 AH, Whatman Inc., NJ, USA) using a MASH II cell harvester(Microbiological Ass., Bethesda, MD, USA). The ^{3}H-thymidine incorporation was determined by counting the radioactivity of the filter discs in a Tri-Carb liquid scintillation counter (Packard Instrument Co.).

Assessment of Protein Synthesis

Twentyfour hours before harvest, the microplates were centrifuged, the cells washed once with leucine free RPMI 1640, and the pellet resuspended in leucine free culture medium supplemented with 20 mCi/ml ^{3}H-leucine(specific activity 53 Ci/mmol, The Radiochemical Centre, Amersham). No further mitogen was added at this time. After 24 hours at 37^{0} C, the plates were centrifuged and replicate supernatants were collected pooled and frozen at -40^{0} C before determination of their Ig content. Cells were then collected on glass filter paper and the ^{3}H-leucine incorporation was determined by counting the radioactivity of the filter discs. For determination of protein secretions the supernatants were precipitated by trichloroacetic acid(5%(w/v) final concentration).

Measurements of Ig Secretion

The assay for the measurement of Ig secretion was performed in microplates: 50 µl of the supernatant(see above) was mixed with 50 µl of monospecific sheep antihuman Fc serum; either anti-Fc of IgG, or anti-Fc of IgA, or anti-Fc of IgM. After 60 min. incubation at room temperature, the mixture was supplemented with 50 µl of a solution containing 30 µgr of either IgG, IgA or IgM purified myeloma protein. These purified myeloma proteins were added in large excess in order to achieve a maximal precipitation efficiency in each assay. All assays were performed in triplicate. The myeloma proteins and the corresponding antisera were prepared as previously described(6,7). The antisera were used at a dilution providing antibody excess as determined by precipitation assays. Their specificity has been assessed by a combination of Ouchterlony and immunoelectrophoresis(14). The plates were then sealed and incubated

overnight at room temperature. The precipitates were collected on glass filter paper(Whatman GF/B) and washed with cold phosphate buffered saline using a Skatron(Flow Lab.) cell harvester. The dried discs were first treated with 200 µl Soluene II(Packard Instrument Co.) before addition of 7 ml toluene scintillation fluid. For each assay, the non-specific binding was measured on supernatants from heat-killed cells, processed under exactly the same conditions. After subtraction of the non-specific binding(mean ± standard deviation), the results were expressed in cpm per 250,000 cells originally present in the culture.

RESULTS

Mitogenic Responses to PHA, ConA and PWM

The ^{3}H-thymidine incorporation into both PHA and ConA stimulated cells is inhibited at concentrations of EHNA exceeding 10 µM(fig. 1a and 1b). However, in the ConA stimulated cultures the inhibitory effect of EHNA is more pronounced. Adenosine(10 µM) potentiates these effects but again the ConA stimulated cultures seem more sensitive than the PHA stimulated cultures(fig. 1a and 1b). In the presence of 1 µM deoxyadenosine hardly any thymidine incorporation at all EHNA concentrations tested is observed. EHNA inhibits the proliferative response of the PWM stimulated cells to an extent comparable to the ConA stimulated cultures (fig. 1b and 1c). This inhibitory action is strongly potentiated by adenosine and even more by deoxyadenosine.

Parameters of T Lymphocyte Activation

PHA stimulation was studied in more detail, using ^{3}H-thymidine incorporation as a parameter for proliferative response and ^{3}H-leucine incorporation as a parameter for protein biosynthesis. Labelling of both intracellular and excreted proteins was inhibited at EHNA concentrations higher than 10 µM(fig. 2a). Adenosine and deoxyadenosine increased this inhibitory effect. In the presence of adenosine(10 µM) the proliferative response was effected at EHNA concentrations above 10 µM. With deoxyadenosine(1 µM) significant inhibition was already observed at an EHNA

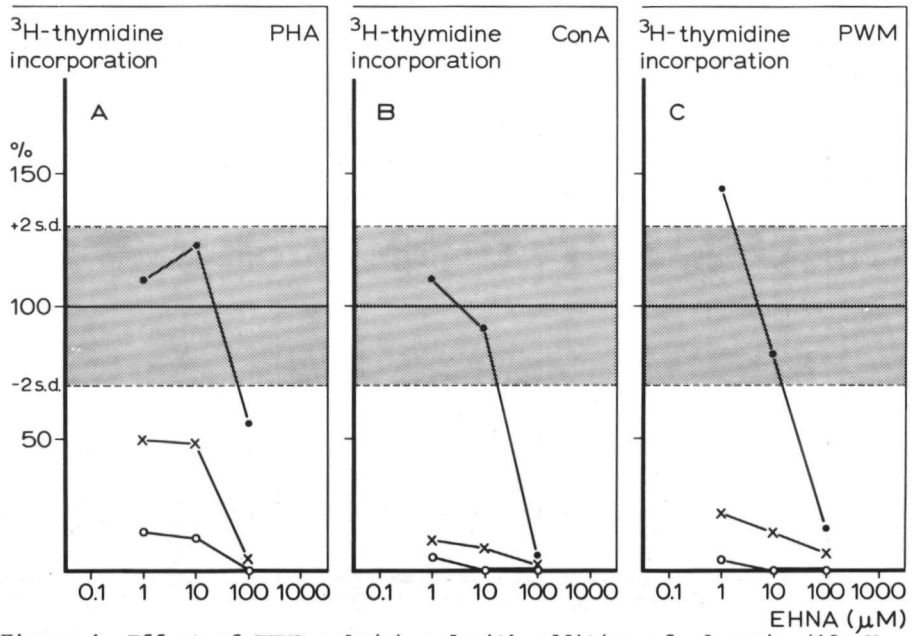

Figure 1: Effect of EHNA only(●) and with addition of adenosine(10 μM; x) or deoxyadenosine(1 μM; o), on the proliferative response of human peripheral blood lymphocytes stimulated by PHA, ConA and PWM. The means (s.d. <15%) of three experiments carried out in three-fold are indicated. The hatched area represents the mean normal control value ± two standard deviations.

concentration of 1 μM(fig. 2a). In PHA stimulated cultures EHNA affected intracellular ^{3}H-leucine incorporation more profoundly than the ^{3}H-thymidine incorporation: at 10 μM EHNA, protein synthesis was clearly depressed, whereas ^{3}H-thymidine incorporation was not(fig. 2b and 2a). Addition of 10 μM adenosine greatly enhanced the inhibitory effect of EHNA on ^{3}H-leucine incorporation. Deoxyadenosine(1 μM) abolished leucine incorporation at EHNA concentrations above 1 μM, whereas at 0.1 μM EHNA no significant inhibition was observed(fig. 2b). The labelling of excreted proteins(fig. 2c) seemed less affected by EHNA than the labelling of intracellular proteins. On the contrary at 0.1 μM EHNA even increased amounts of radioactivity were measured in the excreted protein fraction (fig. 2c). An inhibitory effect of EHNA alone was only seen at the highest concentration used(100 μM). Even more clearly as was the case with thymidine incorporation, adenosine(10 μM) and deoxyadenosine(1 μM) potentiated the effect of EHNA.

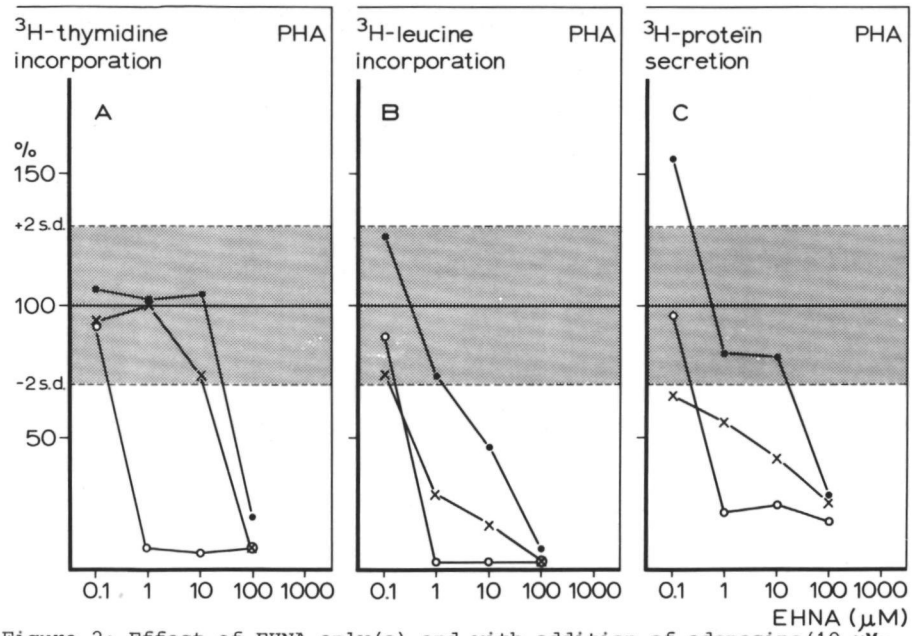

Figure 2: Effect of EHNA only(●) and with addition of adenosine(10 μM; x) or deoxyadenosine(1 μM; o), on the proliferative response, protein synthesis and protein secretion of human peripheral blood lymphocytes stimulated by PHA. The means(s.d. <15%) of three experiments carried out in three-fold are indicated. The hatched area represents the mean normal control value ± two standard deviations.

Parameters of T cell Dependent B Lymphocyte Activation

In this set of experiments PWM and SpA were used as T cell dependent B lymphocyte mitogens. Since essentially the same results were obtained with both mitogens, only the data on PWM are reported. Next to ^{3}H-thymidine and ^{3}H-leucine incorporation, the secretion of ^{3}H-IgG, ^{3}H-IgA and ^{3}H-IgM was studied in the PWM stimulated cultures. Only the data on IgG are reported, since IgA and IgM measurements essentially showed the same results.

In PWM stimulated cultures, EHNA alone inhibits IgG synthesis at concentrations higher than 100 μM. Both adenosine(10 μM) and deoxyadenosine(1 μM) show a strong synergistic action(fig. 3c). As IgG production is not necessarily related to lymphocyte proliferation, the influence of the above compounds on ^{3}H-thymidine and ^{3}H-leucine uptake in parallel

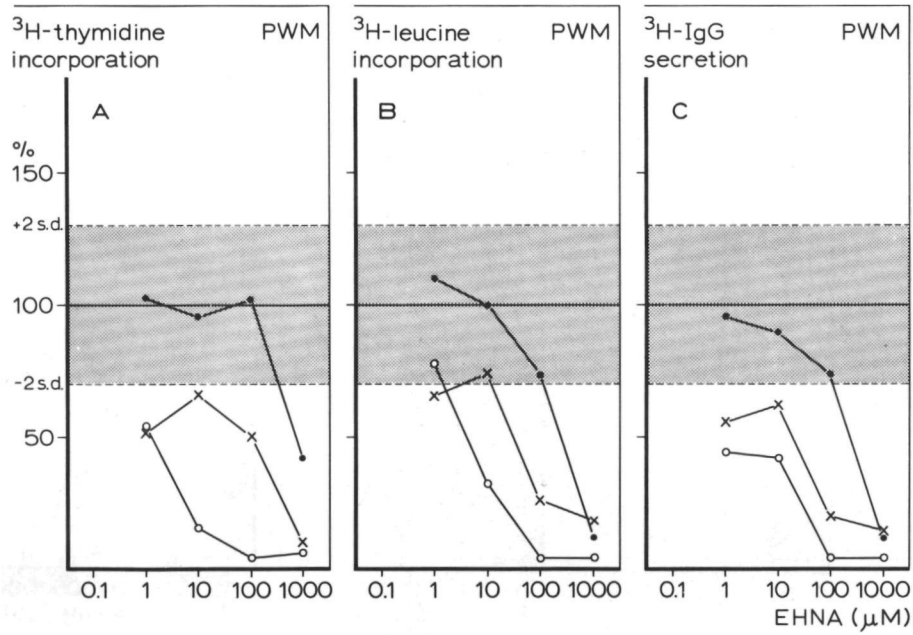

Figure 3: Effect of EHNA only(●) and with addition of adenosine(10 μM; x) or deoxyadenosine(1 μM; o), on the proliferative response, protein synthesis and IgG secretion of human peripheral blood lymphocytes stimulated by PWM. The means(s.d. <15%) of three experiments carried out in three-fold are indicated. Th hatched area represents the mean normal control value ± two standard deviations.

cultures has been assessed. The results indicate that parallelism exists between the inhibition of labeled IgG secretion and the intracellular thymidine and leucine incorporation(fig. 3a, 3b and 3c). In PWM stimulated cultures, EHNA alone showed only at 1000 μM clear inhibitory effects on both thymidine and leucine incorporation. As in the PHA stimulated cultures(fig. 2a en 2b), the presence of EHNA and 10 μM adenosine enhanced these effects. In the presence of 1 μM deoxyadenosine, thymidine and leucine incorporation were even more reduced; at EHNA concentrations of 100 and 1000 μM incorporation levels were at the limit of detection (fig. 3a and 3b).

Using increasing amounts of adenosine with 1 μM EHNA, incorporation of thymidine and leucine and secretion of IgG appeared to be dose dependent(fig. 4a, 4b and 4c). Note that in cultures without any EHNA added, adenosine had no significant effect on these three parameters. In

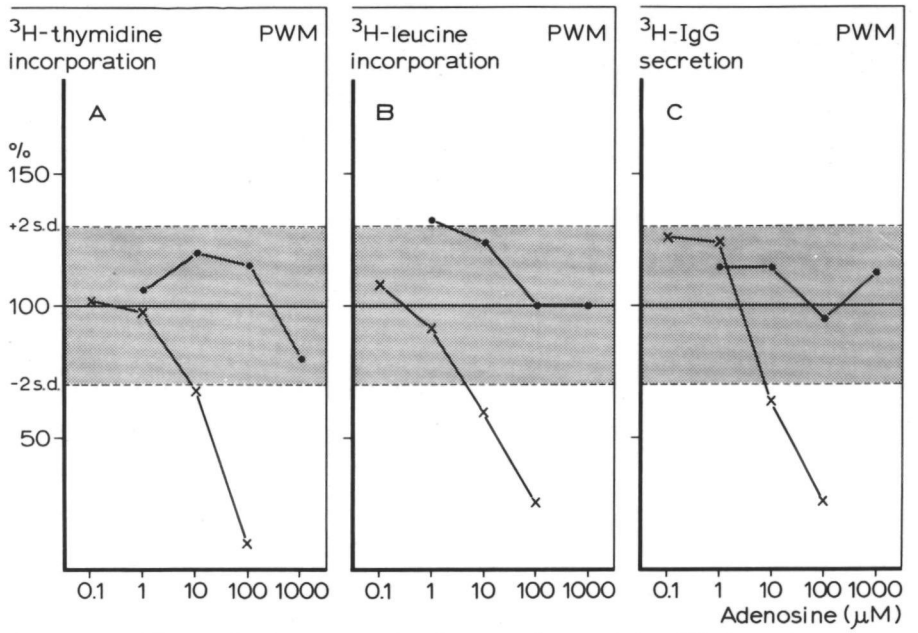

Figure 4: Effect of adenosine with(x) or without(•) addition of EHNA (1 μM) on the proliferative response, protein synthesis and IgG secretion of human peripheral blood lymphocytes stimulated by PWM. The means (s.d. <15%) of three experiments carried out in three-fold are indicated. The hatched area represents the mean normal control value ± two standard deviations.

parallel experiments deoxyadenosine alone(1 to 1000 μM) had no striking effect on thymidine and leucine incorporation, nor on ^{3}H-IgG secretion (fig. 5a, 5b and 5c). In the presence of 1 μM EHNA however, a dramatic inhibition of thymidine uptake was already observed at 0.1 μM deoxyadenosine. This was in contrast with the biphasic effect on the ^{3}H-leucine and ^{3}H-IgG secretion. At 0.1 and 1 μM deoxyadenosine, labelling was significantly increased in the presence of 1 μM EHNA, whereas at 10 and 100 μM deoxyadenosine a striking decrease of leucine incorporated and ^{3}H-IgG secreted was seen. In cultures performed in the presence of 0.1 μM deoxyadenosine and 1 μM EHNA an inhibition of thymidine uptake was coexistent with an increase of ^{3}H-leucine incorporation and IgG secretion(fig. 5).

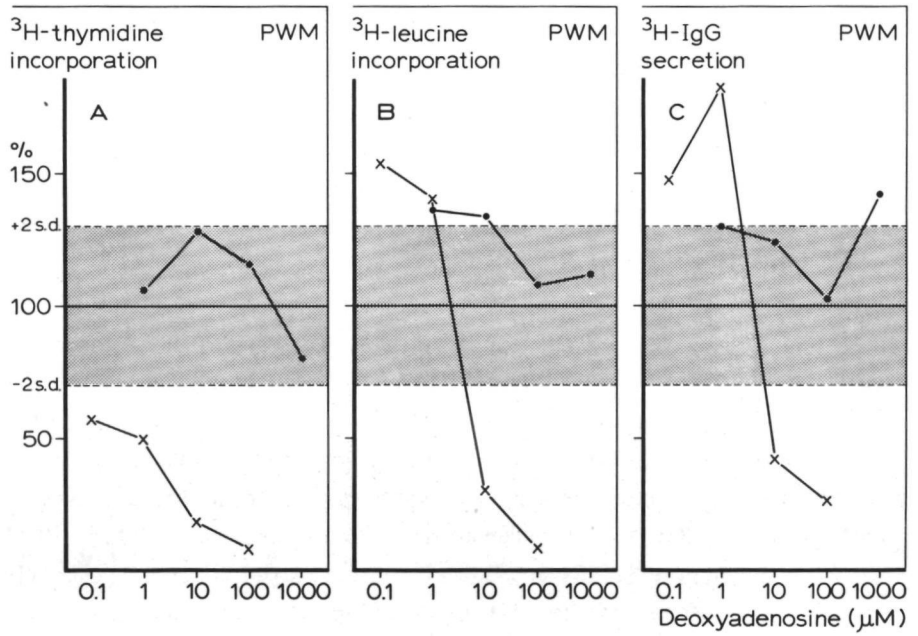

Figure 5: Effect of deoxyadenosine with(x) or without(●) addition of EHNA(1 μM) on the proliferative response, protein synthesis and IgG secretion of human peripheral blood lymphocytes stimulated by PWM. The means(s.d. <15%) of three experiments carried out in three-fold are indicated. The hatched area represents the mean control value ± two standard deviations.

DISCUSSION

The present results confirm and extend previous findings: in the presence of an ADA inhibitor, T cell mitogenic responses are inhibited by adenosine and deoxyadenosine(3,13,18). When comparing thymidine and leucine incorporation as parameters, the latter seems a more sensitive index(fig. 2a and 2b; ref. 3).

Inhibiting ADA activity with varying amounts of EHNA will eventually lead to an accumulation of a certain amount of adenosine and deoxyadenosine. Apparently this has no detrimental effect on thymidine and leucine uptake in the PWM stimulated cultures. On the other hand when inhibiting ADA with a fixed EHNA concentration and adding extra adenosine or deoxyadenosine, the accumulation of these compounds and their derivatives might lead to effects mediated by the different mechanisms mentioned in

the introduction, depending on the respective concentrations.

The present results show that ^{3}H-thymidine incorporation in cultures stimulated by ConA and PWM is more sensitive to the inhibitory effects of EHNA and (deoxy)adenosine than in the PHA stimulated cultures(fig. 1). These results suggest that subsets of T cells are differently affected. Indeed it has been shown that PHA and ConA stimulate different though overlapping T cell subsets(10). ConA stimulates both suppressor and helper cells and PHA mainly stimulates T-helper cells. Due to this fact, one might speculate that T-suppressor cells are more vulnerable as compared to T-helper cells to the inhibitory effects of adenosine and deoxyadenosine in combination with EHNA. It should be pointed out that the relative amounts of thymidine and leucine incorporated may vary between different experiments. For instance in comparable experiments, fig. 1a and fig. 2a, the thymidine incorporation in the presence of 10 μM EHNA and 10 μM adenosine is 75% and 50% of the untreated control, respectively. However, within one experiment the variation was never more than 10 to 15%. This stresses the importance of a precise definition of both the methods used to purify mononuclear cells and the culture conditions(8). Among these the nature of the serum is particularly critical. Indeed it is known that serum differs in adenosine and ADA content, even after heat inactivation.

Interesting is the observation of the parallelism observed in the PHA stimulated cultures between the effects of these compounds on thymidine incorporation on one hand and leucine incorporation on the other hand. However, inhibition is more pronounced in the latter situation(fig. 2). Still more interesting is the observation of a facilitating effect of a very low concentration of EHNA(0.1 μM) on the protein secretion. At present no explanation can be given for this finding.

From the data in fig. 3c and 4c, it is evident that the combination of EHNA and adenosine inhibits the ^{3}H-IgG production by PWM stimulated cells. This inhibition might be secondary to a blockade of either the B cell, or to enhancement of T-suppressor activity.

As opposed to adenosine, deoxyadenosine exerts a biphasic effect (fig. 5). At low concentrations and in the presence of EHNA and 1 μM

deoxyadenosine it enhances labeled IgG secretion, whereas higher concentrations have an inhibitory effect. This enhancing effect is associated with a reduction in thymidine uptake. As it is known that DNA synthesis is necessary for suppressor activity and not for T-helper activity(12), these data could be interpreted as indicating a blockade of suppressor cells at deoxyadenosine concentrations below 1 μM. The above hypothesis is amenable to experimental testing, namely by comparing the effects of adenosine to those of deoxyadenosine in the presence of EHNA on purified preparations of T-helper and T-suppressor cells.

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chapter 11

PURINE METABOLISM IN RELATION TO LEUKEMIA AND LYMPHOID CELL DIFFERENTIATION

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INTRODUCTION

After Giblett and associates(65) in 1972 had postulated a causal relationship between a genetic deficiency of an enzyme of purine metabolism, adenosine deaminase(ADA), and a combined deficiency of T and B cell function, a serious effort has been made to elucidate the role of purine metabolism in immune function(120,136,170). The metabolic basis for immunodeficiencies, the selective sensitivity of the immune system for deficiencies of enzymes of purine metabolism and the different susceptibility for deoxynucleosides between T and B cells, have been subject of extensive studies during the last decade.

These studies were mainly carried out in in vitro systems. Mouse 3T6 fibroblasts(69), human lymphoblastoid cell lines(104) as well as freshly isolated cells(81) are some examples of these in vitro systems. Both animal(177) and human(133) in vivo studies were also done for investigating the relation between purine metabolism and the immune system. These studies provided evidence that purine metabolism plays an essential role in the development of the immune sytem. This was also illustrated by the finding, that different activity levels of purine metabolizing enzymes were found in various stages of lymphoid differentiation.

Studies on purine metabolism in lymphocytic leukemia showed that the enzymatic make-up of lymphoid cells in certain types of lymphocytic leukemia was comparable to the enzymatic make-up of lymphoid cells in certain stages of their development. Therefore, this paper discusses the intimate realtionship between immunodeficiency diseases, normal lymphocytic differentiation, lymphocytic leukemia and purine metabolism. In scheme 1, relevant enzymatic reactions and metabolites of purine and pyrimidine metabolism are depicted(scheme 1 is located at page 179).

1. INBORN ERRORS OF PURINE METABOLISM AND IMMUNODEFICIENCY DISEASE

Adenosine Deaminase Deficiency

ADA catalyzes the deamination of adenosine and deoxyadenosine to inosine and deoxyinosine(scheme 1, fig. 1). Giblett et al.(65) were the first to report absence of ADA activity in red cell lysates from two young unrelated girls. These girls had a clear dysfunction of T and B cells, which was manifested by recurrent infections, delayed hypersensitivity reaction, poor in vitro mitogenic stimulation, low IgG and IgM plasma levels, lack of blood group antigens and other defects. After this first report on ADA deficiency related with severe combined immunodeficiency disease(SCID) several other patients were described(115,116, 162,190). It appeared that 22% of the SCID patients was ADA deficient (89). Skeletal abnormalities next to the presence of a rudimentary thymus with no clear architecture and small lymph nodes were reported (190). ADA activity levels in lymphocytes from SCID patients who had no detectable ADA activity in their red blood cells, were less than a few percent of normal lymphocytic ADA activity(76,114). Studies on normal tissues and tissues from SCID patients revealed that in the normal tissues ADA activity was highest in the thymus(87). ADA deficient SCID patients showed only residual ADA activities in the tissues studied (85,87,148,178). The relative residual ADA activity however, was found to be highest in the liver(31%) and not in lymphoid organs such as thymus(0.2%), spleen(1.1%) and lymph node(3.6%).

In one study on red cell lysates from a SCID patient, evidence was presented that the deficient ADA activity was due to a genetically

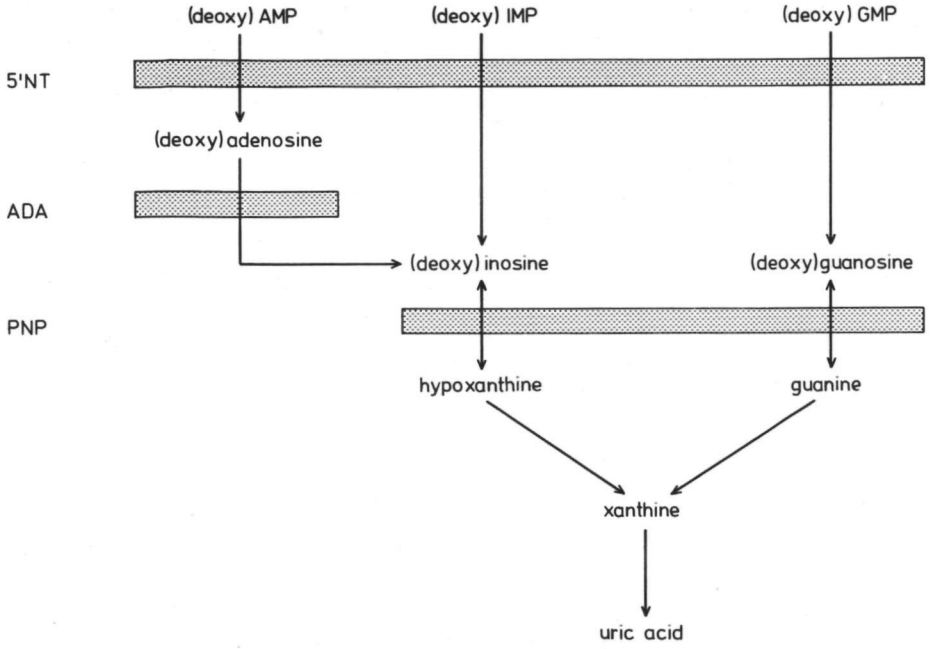

Figure 1: Purine enzyme defects associated with immunodeficeincy. ADA= adenosine deaminase. 5'NT=purine-5'nucleotidase. PNP=purine nucleoside phosphorylase.

programmed production of an ADA inhibitor(173). General agreement however, exists that the inherited deficiency of ADA is caused by a structural mutation(44,86). In a case were no tissue or tissue culture material was available from a child who died of SCID, the mutant ADA was investigated in cell hybrid clones, derived from one of the heterozygous parents(76). In this study it was shown that a structural mutation on chromosome 20 was causally related to the SCID. Similar data were obtained in a study on fibroblasts from 4 SCID patients with red blood cell ADA deficiency. The residual enzyme activities in fibroblast lysates from these patients ranged up to 27% of the mean control value (85).

Two cases with residual ADA activity have been reported with an apparently normal immune function. The so called Kung boy, who had strongly decreased ADA activity in red blood cells but only mildly decreased ADA activity in leukocytes and cultured fibroblasts, showed

normal levels of serum immunoglobulins, normal mitogenic stimulation of peripheral blood lymphocytes and normal numbers of T and B lymphocytes(96). A 20 month old male child white normal white blood cell counts and normal responses to mitogen but with less than 1% of normal red blood cell ADA activity was described more recently(88). In lymphoid cell lines from this patient ADA activity was about 30% of normal values and about 50-fold higher than in lymphoid cell lines from ADA deficient SCID patients. This patient is resembled by a patient, described by Perignon et al.(127) who had less than 1% red blood cell ADA activity, but a residual ADA activity in the lymphocytes. These data on ADA deficiency in relation to SCID indicate that the nature of the mutations that lead to ADA deficiency must be rather heterogeneous. In this context the term "deficiency" is meant to indicate a mutant ADA activity resulting in SCID.

Altered levels of purine metabolites have been described in cells and body fluids from SCID patients. Elevated levels of adenosine and deoxyadenosine, both substrates for ADA, have been observed in urine (49,106). In plasma of these patients, elevated levels of adenosine were reported(38,106,117,130). Levels of phosphorylated derivatives of adenosine and deoxyadenosine, namely ATP and deoxyATP, were reported to be increased in red blood cells(38,43,49,92,117) as well as in mononuclear cells from ADA deficient SCID patients(49,147). However, also ATP values within the normal range were reported(2). The presence of elevated levels of adenosine and deoxyadenosine and their nucleotides, ATP and deoxyATP, in body fluids, erythrocytes and mononuclear cells from ADA deficient SCID patients indicated that these purines might play an essential role in the pathophysiological mechanism(s) leading to the immune dysfunction. A review on ADA deficiency and SCID has been published by Thompson and Seegmiller(170).

Purine Nucleoside Phosphorylase Deficiency

Purine nucleoside phosphorylase(PNP) catalyzes the conversion of inosine and deoxyinosine to hypoxanthine and (deoxy)ribose-1-phosphate and of guanosine and deoxyguanosine to guanine and the deoxyribose moiety(scheme 1, fig. 1). In several patients with severely defective

T cell immunity but with apparently normal B cell function, a deficiency of purine nucleoside phosphorylase was found(4,66,72,156). In PNP deficient patients generally lymphopenia, decreased PHA responses and recurrent infections were noted. The number of cells bearing surface immunoglobulins(sIg) and the formation of antibodies were found to be normal(141). Kinetic studies in erythrocytes of two PNP deficient brothers suggested that PNP deficiency is caused by a mutation in the structural gene(58). The heterogeneity in the expression of the disease may be accounted for by different residual activities caused by diferent mutations.

Inosine concentrations were found to be elevated in plasma(51,157) and relatively high inosine and guanosine concentrations(51,141,157) as well as deoxyinosine and deoxyguanosine(36) were detected in urine. In contrast low plasma levels of uric acid were observed(36,141,157). Guanosine and deoxyguanosine can be phosphorylated by a kinase to GMP or deoxyGMP, which are subsequently converted to GTP or deoxyGTP. The substrates for these enzymatic reactions(scheme 1) are present at relatively high levels in body fluids of PNP deficient patients. Therefore, elevated levels of GTP and deoxyGTP in red and white blood cells of these patients can be expected. Indeed elevated deoxyGTP and GTP concentrations have been reported in erythrocytes of these patients(37,113). Since there is hardly any production of the free purine bases hypoxanthine and guanine in PNP deficiency, the formation of GMP and IMP through the salvage enzyme hypoxanthine-guanine phosphoribosyltransferase(HGPRT, scheme 1) will not be of any significance. This might lead to elevated levels of PRPP, the cosubstrate of this salvage reaction. Indeed elevated PRPP levels were described in the erythrocytes, but not in the fibroblasts, from a PNP deficient child(36)

In lyzed lymphocytes of one single patient with common variable immunodeficiency, decreased PNP(15% of control value) activity , and a decreased activity(15% of control value) of purine-5'nucleotidase was described(125). Although the number of E-rosette forming cells was rather high, a clearly depressed response of the T cells on phytohaemagglutinin(PHA) was observed. B cells were virtually absent and serum Ig levels were clearly decreased. The activity of ADA in this patient appeared to be in the normal range. It could not be shown whether this

case represents causal relation between purine metabolism and immune dysfunction: in the case of a maturational block the altered enzyme activities might merely reflect a stage of maturation in which these cells were arrested(paragraph 3).

The same might be said about the decreased PNP activity described in T cells from a patients with the autoimmune disease systemic lupus erythematosus(107). The alterations in the balance of several subsets of white blood cells might be the cause of this, only slightly, decreased PNP activity. Any causal relation cannot be assumed from the data reported(107).

Purine-5'nucleotidase Deficiency

Purine-5'nucleotidase(5'NT) catalyzes the first enzymatic step in the degradation of purine nucleotides(e.g. AMP, deoxyAMP, GMP), leading to the formation of the corresponding nucleosides(scheme 1, fig. 1). Johnson et al.(97) and Edwards et al.(52) described decreased activities of 5'NT in patients with X-linked agammaglobulinaemia(Bruton type, XLA), measured both in lymphocyte lysates and in intact lymphocytes. These findings were confirmed and extended(186). It was shown that 5'NT activities were up to 25% of control value in XLA patients when using different mononucleotide substrates. ADA and PNP activities and several other purine enzyme activities were normal in these patients.

The data described above were doubted by other authors(50). It was postulated that the increased amount of monocytes in XLA patients, which lack or have only low 5'NT activity(53,98), was responsible for the decreased 5'NT activity. The 5'NT activity in lymphocytes would be in the normal control range(50). However, this hypothesis was disproven in experiments on rosetting peripheral blood mononuclear cells(53). These E-rosetting cell populations in XLA patients, which contained less than 2% monocytes, showed a clearly decreased ecto-5'NT activity as compared to control peripheral blood rosetting cells. Whether this decreased activity should be ascribed to the B or T cell population or to both, could not be concluded according to the authors. More recent studies (39,54,169) indicated that both T and B cells have decreased 5'NT

activities in XLA. The presence of normal 5'NT activity in fibroblasts and T-colony lymphocytes indicate that a mutation in the structural gene for 5'NT is not likely. The findings of low 5'NT activity in XLA patients might be the reflection of a maturational disturbance of their lymphoid cells. Accumulation of deoxyribonucleotides in white and red blood cells of XLA patients has not been observed(113).

2. PATHOPHYSIOLOGICAL MECHANISMS OF LYMPHOTOXICITY

Elevated levels of adenosine and deoxyadenosine have been found in body fluids from ADA deficient patients with SCID(38,49,106,117,130,158). Intracellular levels of ATP, deoxyATP and cyclic AMP(cAMP) were found to be increased(38,43,49,92,117,147,170). In PNP deficient individuals increased levels of guanosine, deoxyguanosine, inosine and deoxyinosine were noted(36,141). Also elevated intracellular levels of deoxyGTP and PRPP have been reported in different cells of these patients(36,37,113). With these findings in mind, it is evident that much research has been focussed on the metabolic fate and the enzymatic handling of these compounds. Biochemical and biological studies have been performed in in vitro models and in human tissues. Five possible mechanisms that might lead to immune dysfunction have been proposed. Parameters as proliferative capacity, protein synthesis and immunocompetence have been investigated. Main attention will be given to ADA and PNP deficiency, since these "inborn errors of metabolism" have most extensively been studied.

A. Adenosine Induced Pyrimidine Starvation

Based on the finding that adenosine caused elevated levels of purine nucleotides and reduced levels of pyrimidine nucleotides, Green and co-workers postulated that adenosine toxicity might be mediated by pyrimidine nucleotide starvation(69,93). Comparable results were obtained in the human lymphblastoid cell line MGL-5, that was established from blood cells of a patient with infectious mononucleosis. When culturing this cell line with adenosine at concentrations higher than 10^{-6} M, growth was strongly affected. This effect was not seen when uridine was provided as an extra source of pyrimidines(69,93).

This phenomenon indicated that indeed the lack of pyrimidines mediated the adenosine induced toxicity.

Not only increased purine nucleotide and decreased pyrimidine nucleotide pools were observed, but also an accumulation of orotic acid was found to be induced by adenosine(93). Adenosine is capable of trapping Pi by phosphorylation to its nucleotides. Under physiological conditions the decrease of intracellular Pi concentrations is accompanied by the inhibition of PRPP formation(paragraph 2B). Thus the accumulation of orotic acid might be explained by the ability of adenosine to limit the intracellular concentration of PRPP, a substrate for orotate phosphoribosyltransferase(128). This is not in accordance with the findings of Snyder et al.(77,167), who found mutants of the WI-L2 lymphoblast cell line deficient in adenosine kinase(AK) to be as sensitive to adenosine as the AK positive cell line. This suggested that adenosine does not need to be converted to its nucleotide in order to cause pyrimidine nucleotide starvation.

Some arguments can be given to doubt a major role of adenosine induced pyrimidine starvation in the toxic mechanism(s), leading to immune dysfunction.

1. Only mildly elevated levels of adenosine in plasma of ADA deficient patients have been reported. These levels were below 5 μM (38,117), whereas pyrimidine starvation induced toxicity occurred at adenosine concentrations of 10 μM or higher.
2. Concentrations of the pyrimidine nucleotides UTP and CTP as well as deoxyTTP and deoxyCTP were found to be elevated in lymphocytes from ADA deficient SCID patients(92,147). The elevation of deoxyTTP and deoxyCTP seems to be contradictory with the inhibition of ribonucleotide reductase(paragraph 2D). This finding may be explained by the erythrocyte transfusions given to this child(92).
3. ADA deficient children do not excrete elevated amounts of orotic acid(118,158).

B. PRPP Starvation

Adenosine reduces the intracellular PRPP concentration in erythrocytes(59,128) and in human lymphoblasts(165,166). As PRPP is an important substrate for purine salvage(HGPRT and adenine phosphoribosyltransferase, APRT; scheme 1) as well as for purine and pyrimidine(orotate phosphoribosyltransferase, OPRT; scheme 1) de novo synthesis, decrease of PRPP levels might have consequences for the absolute and relative amounts of the different purine and pyrimidine nucleotides in the cell.

Nucleosides are capable of increasing or decreasing the intracellular PRPP concentrations in vitro. When Pi is abundantly available, nucleosides stimulate PRPP synthesis. When Pi is limiting, nucleosides inhibit PRPP formation. A decrease of the physiological intracellular Pi concentration accompanies the inhibition of PRPP formation(128). Nucleosides are capable of reducing intracellular Pi levels. This is related both to nucleosides that are predominantly degraded to the purine base and to those that are substantially phosphorylated. Both these pathways trap Pi. It was shown that when erythrocytes were incubated under physiological Pi conditions the PRPP levels drop upon addition of nucleosides(128). However, in packed erythrocytes of a PNP deficient patient elevated levels of PRPP were found(36); this as a consequence of blocked purine salvage pathways and a sparing effect of PRPP. Therefore it becomes rather unlikely that in PNP deficiencies PRPP is an important mediator for the toxic effects. Further investigations are warranted.

C. Inhibition of S-Adenosyl Homocysteine Hydrolase(SAHH) by Adenosine and Deoxyadenosine

As mentioned before, in body fluids of ADA deficient patients increased levels of adenosine and deoxyadenosine were found(49,106, 117,130). From experiments with mutant AK deficient lymphoblastoid lines it appeared that adenosine needs not to be phosphorylated to exert inhibitory effects(167). Therefore eventual inhibition of SAHH, by adenosine and/or deoxyadenosine seemed to be a process that needed

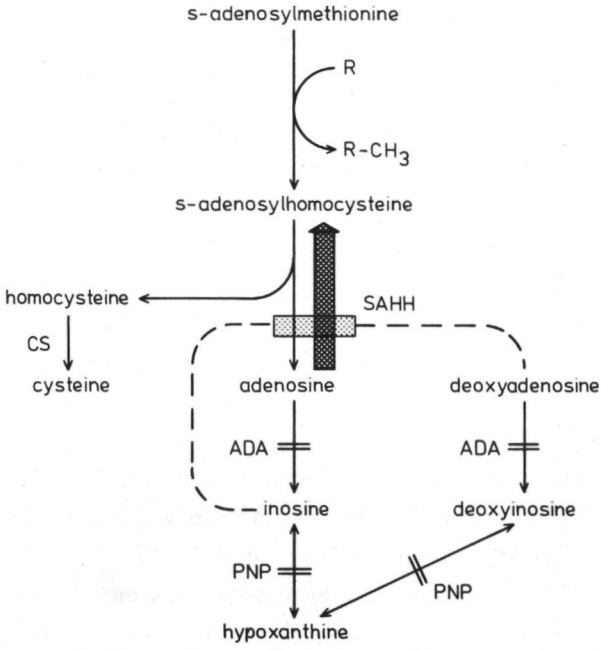

Figure 2: The metabolic pathway of s-adenosylhomocysteine in ADA and PNP deficiency. R=methylreceptor like RNA, DNA, lipids, etc. SAHH= s-adenosylhomocysteine hydrolase. CS=cystathione synthetase. The broken lines represent irreversible inhibition of SAHH (modified from 104).

investigation.

SAHH catalyzes the reversible hydrolysis of s-adenosylhomocysteine(AdoHcy) to adenosine and homocysteine(fig. 2, 48). Under physiological conditions this reaction favours the catabolic direction (hydrolysis of AdoHcy in adenosine and homocysteine). The reaction products adenosine and homocysteine are readily removed by adenosine deaminase and cystathione synthetase(fig. 2), respectively. The equilibrium constant however, is most favourable for the anabolic direction(48). In experiments where adenosine(1 μM) was incubated with crude lymphoblasts extracts, adenosine was preferently recovered from one peak after gelfiltration of the lysate. This protein peak had neither ADA nor AK activity. However, this peak appeared to have AdoHcy synthesizing capacity(78). It was concluded that SAHH binds adenosine with high affinity. In several systems it was shown, that addition of adenosine increased the level of AdoHcy. Mice

intraperitoneally injected with different concentrations of adenosine and homocysteine produced a dose related increase of AdoHcy levels in blood, liver and brain(145). Incubation of WI-L2 lymphoblasts and AK deficient mutants with adenosine and the ADA inhibitor EHNA leads to striking elevations of AdoHcy in these cells(104). Not only adenosine, but also deoxyadenosine appears to have an AdoHcy elevating effect in human lymphoblasts(79,80) and in eryhtrocytes(81). In this same study the binding of deoxyadenosine to SAHH appeared to cause an irreversible inactivation of the catalytic activity. This irreversible inhibition was also seen with adenosine(33).

Among the nucleosides accumulating in PNP deficiency only inosine was able to cause a phosphate dependent irreversible inactivation of human SAHH(82). This probably explains the low levels of SAHH found in hemolysate from PNP deficient patients(82,100). Hypoxanthine facilitated the SAHH inactivation by inosine. Hypoxanthine probably shifts the equilibrium of the PNP reaction into the direction of inosine formation. This might explain the decreased activity of SAHH in Lesch-Nyhan patients, who lack HGPRT activity. Inhibition of SAHH will lead to increased levels of AdoHcy, which is a potent inhibitor of numerous s-adenosylmethionine dependent methyl transfer reactions(81,99,146). Such inhibition appears to account for nucleotide independent adenosine toxicity to mouse(103) and human lymphoblasts(104).

The inhibition of SAHH is an event that may also occur in ADA deficient patients, where adenosine and deoxyadenosine is accumulated. Recently it has been reported(119) that in an ADA deficient SCID patient the urinary excretion of AdoHcy is elevated. In an ADA deficient patient, who was not immunodeficient, no elevated urinary excretion of AdoHcy was reported. However, also SCID patients with apparently normal ADA activity with elevated AdoHcy excretion have been found(119). Although adenosine and/or deoxyadenosine induced inhibition of methylation pathways may play an important role in the mediation of the toxic defects in immunodeficiency, the exact role of this process has still to be elucidated.

D. Inhibition of Ribonucleotide Reductase by DeoxyATP and DeoxyGTP

Much attention has been focussed on the allosteric inhibition of ribonucleotide reductase by deoxyribonucleotides. This enzyme catalyzes the conversion of ribonucleotides, at the diphosphate level, to deoxyribonucleotides; the conversions of ADP to deoxyADP, GDP to deoxyGDP, UDP to deoxyUDP and CDP to deoxyCDP(fig. 3). Since this is the only route known for synthesis of deoxynucleotides in mammalian cells(122,140), its action is essential for DNA synthesis. Using highly purified calf thymus ribonucleoside diphosphate reductase, it was shown that deoxyATP at a concentration of 5 μM inhibits 50% of the ADP, GDP, CDP and UDP reduction(56). The reduction of GDP, CDP and UDP was inhibited by deoxyGTP at concentrations of 50 to 100 μM. In ADA and PNP deficient patients, deoxyadenosine and deoxyguanosine are accumulated, respectively. These deoxyribonucleosides can be phosphorylated to monodeoxyribonucleotides which subsequently increase the intracellular levels of deoxyATP and deoxyGTP. Elevated deoxyATP and deoxyGTP levels have been found in cells of immunodeficient patients(paragraph 1). Inhibition of ribonucleotide reductase by either one of these compounds(fig. 3) will cause a disbalance of the intracellular deoxyribonucleotide pools and thus impairment of DNA synthesis.

The phosphorylating capacity of the thymus, spleen and peripheral lymphocytes is high when compared with other tissues(26). In experiments with the human T lymphoblast MOLT-4 and the B lymphoblast MGL-8 cell lines it was seen that, when culturing both cell lines with deoxyadenosine or deoxyguanosine, the cell growth was much more inhibited in the T cell line than it was in the B cell line(121). Together with this selectivite toxicity a selective accumulation of deoxyATP and deoxyGTP in the T lymphoblasts was observed(121). In comparable experiments with deoxyadenosine in , partly other, T and B cell lines, this selective toxicity was confirmed(27). However, measuring the deoxyadenosine kinase activity(catalyzing the phosphorylation of deoxyadenosine to deoxyAMP, scheme 1), showed that the explanation of this selectivity could not simply be given by the relative amounts of this enzyme present. Studies carried out in mouse T cell lymphoma lines(30,70) showed that deoxyguanosine,

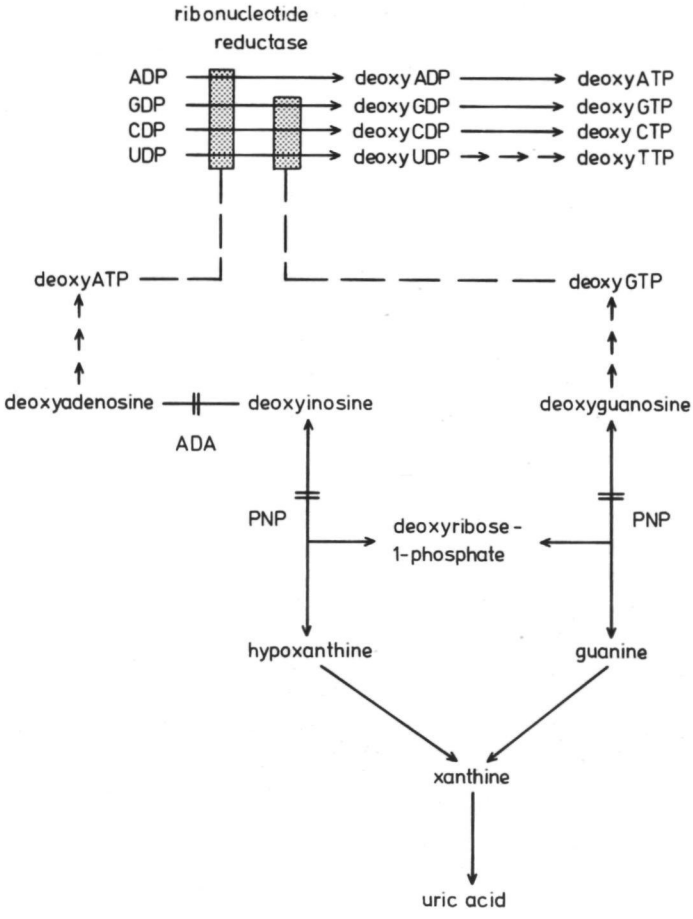

Figure 3: The inhibition of ribonucleotide reductase by deoxyATP and deoxyGTP in ADA and PNP deficiency(modified from 136).

guanosine and deoxyinosine inhibited cell growth. A drop of deoxy-CTP and deoxyATP levels, accompanied by a sharpe rise in deoxyGTP levels, pointed to a mechanism related to inhibition of ribonucleotide reductase(fig. 3).

As indicated above, the higher toxicity for T cell lines could not be explained solely by a significantly higher kinase activity than in B cell lines(27,124). Differences in 5'NT activities between T and B cells might contribute to this selectivity(chapter 5, 169, 183). The most rapid accumulation of deoxyATP upon incubation with

deoxyadenosine was seen in T cell lines in several studies(28,121,188). The phosphorylating capacity of deoxyadenosine did not differ significantly in T and B cell lines(121,188). However, marked differences in the nucleotide degrading capacity were noticed. 5'NT, the enzyme dephosphorylating deoxyAMP and other mononucleotides, to give the corresponding nucleosides, was found to have a much lower activity both in freshly isolated and in cultured T and B cells(chapter 5, 169,183,188). Thus, the more rapid accumulation of deoxyATP might be considered to be the net result of both kinase and nucleotidase activity. This mechanism was not only suggested in the case of deoxyATP accumulation, but also in the case of deoxyTTP(28) and deoxyGTP accumulation(121).

Although the deoxynucleotide toxicity seems rather plausible, several points of criticism have to be made:

1. It is hard to explain the selective toxicity towards T and B cells, only on the basis of differences in the 5'NT activity, an enzyme of which a considerable part of the catalytic activity is located at the outer surface of the cell membrane. Data supporting this doubt have recently been published(18). ADA deficient cell lines, which have relatively high ecto-5'NT activities exhibit a sensitivity to deoxyadenosine intoxication, comparable to ADA deficient cell lines with relatively low ecto-5'NT activities. However, no data are given in this paper on the activity of nucleoside kinases. The recent finding of a soluble deoxynucleotidase in lymphoblasts, that is distinguishable from the membrane bound enzyme(29), might throw new light on this matter.
2. A mutant human lymphoblastoid cell line that lacked deoxycytidine kinase, had less than 10% of the deoxyadenosine phosphorylating capacity as compared to the wild type(176). Deoxycytidine kinase is believed to catalyze the phosphorylation of deoxyguanosine and deoxyadenosine(105). With regard to growth this mutant was as sensitive to the toxic effects of deoxyadenosine as the wild type. A WI-L2 mutant deficient in adenosine kinase activity showed no significant difference in deoxyadenosine phosphorylating activity when measured in extracts as compared to the wild type. However, this mutant was 3-fold less sensitive to deoxyadenosine and accumulated far less deoxyATP from exogenous deoxyadenosine.

A mutant cell line lacking both adenosine and deoxycytidine kinase activities was less sensitive to deoxyadenosine than the adenosine kinase deficient mutant and did not accumulate deoxyATP. It was concluded that although deoxyadenosine phosphorylation was associated with deoxycytidine kinase, the physiologically important deoxyadenosine phosphorylating capacity is associated with the adenosine kinase activity.

E. Adenosine Induced Elevation of Intracellular cAMP

It is known that PHA induced human lymphocyte blastogenesis(25, 161) is inhibited by cAMP. Carson and Seegmiller(25) reported that, when normal peripheral blood lymphocytes were cultured with cAMP or dibutyryl-cAMP(db-cAMP), ^{3}H-thymidine incorporation was inhibited. It was suggested that the action of cAMP was mediated by adenosine to which it has been catabolized. This can be concluded from experiments in which EHNA(a potent inhibitor of adenosine catabolism) enhanced the cAMP effect. Not only lymphocyte blastogenesis could be inhibited by cAMP, also the excretion of antibodies(161) and lymphocyte mediated toxicity(19,168) were affected by this compound. db-cAMP nearly abolished cytotoxicity at a concentration of 1 mM. cAMP also caused a reduction of the concentrations of UTP and CTP in lymphocytes(166). Apparently, through breakdown to adenosine, cAMP is capable of causing a pyrimidine nucleotide starvation in human lymphoblasts. The effect could be reversed by the addition of uridine (25,166).

Adenosine and deoxyadenosine are known to increase intracellular concentrations of cAMP(35,187). As a result of elevated adenosine and deoxyadenosine levels in ADA deficiency, intracellular cAMP levels in cells of the lymphoid system might be increased either as a result of metabolic transformation of adenosine to cAMP or as a consequence of adenyl cyclase activation, the enzyme that catalyzes the conversion of ATP to cAMP.

Whether the elevation of intracellular cAMP plays an important role in the biochemical mechanisms leading to immune dysfunction in

Table 1: Possible Pathophysiological Mechanisms in ADA Deficiency

Accumulation	Consequence
Adenine ribonucleotides	-Pyrimidine ribonucleotide starvation -PRPP starvation
S-adenosylhomocysteine	-Decreased methylation of DNA, RNA, proteins, etc.
Adenine deoxyribonucleotides	-Inhibition of ribonucleotide reductase
cAMP	-Impaired immune response

ADA deficiency, is not yet clear. ADA deficient leukocytes contained a cAMP concentration(6 pmol/10^6 cells) twice as high as in control leukocytes(2.6 pmol/10^6 cells, 147). In the reports described above, severe inhibitory effects of cAMP were seen only at concentrations over 100 µM(25,166). Moreover, mutants from S49 cells(mineral oil induced, T cell lymphosarcomas obtained from Balb/c mice) defective in some component of cAMP action or metabolism, are resistant to killing by isoproterenol, a hormone that increases cAMP levels, whereas they are sensitive to killing by adenosine and EHNA(175). Through these findings it becomes doubtful whether this mechanism is essential in the mediation of toxic effects of ADA deficiency.

In table 1 the possible pathophysiological mechanisms in immunodeficiency disease are summarized. It is not likely that either one of these mechanisms is solely responsible for the immunodeficiencies caused by ADA or PNP deficiency. It is more likely that a combination of any of these mecahnisms may finally result in the immune defect. In view of the moderately elevated concentrations of adenosine(paragraph 2A), the elevated levels of PRPP in PNP deficiency(paragraph 2B) and the moderately elevated levels of cAMP in ADA deficient leukocytes(paragraph 2E), the possibilities discussed under A, B and E in this paragraph, most probably will not play an essential role. Only low concentrations of adenosine and deoxyadenosine appear to be able to inhibit SAHH(paragraph 2C). DeoxyATP(3 µM) inhibits 50% of ribonucleotide reductase activity(paragraph 2D). In conclusion, the inhibition of SAHH and/or ribonucleotide reductase is likely to play a major role in the pathophysiological mechanisms of immunodeficiency disease.

3. ENZYMOLOGICAL ASPECTS OF LYMPHOID CELL DIFFERENTIATION

The lymphocyte part of the immune system in birds and mammals consists of two major differentiation pathways, which have a common origin. Lymphopoietic precursor cells migrate from yolk sac, fetal liver, spleen and bone marrow via the blood stream to the central lymphoid tissues, the thymus and the bursa of Fabricius in birds or its equivalent, fetal liver and bone marrow, in mammals. After having reached the thymus or the bursa equivalent, lymphocyte precursor cells undergo proliferation and differentiation to become immunologically competent lymphocytes. The newly differentiated lymphocytes migrate from the central lymphoid tissues to the peripheral lymphoid tissues(spleen, lymph nodes and gut-associated lymphoid tissues) where.subpopulations populate distinct anatomical locations(55). Peripheral lymphocytes, which have undergone differentiation in the thymus before emigrating to peripheral lymphoid tissues, are referred to as T lymphocytes or T cells. Mature T cells are responsible for cell mediated immunological reactions. Peripheral lymphocytes, which differentiated in the bursa of Fabricius or its mammalian equivalent, are referred to as B lymphocytes or B cells. Mature cells are responsible for antibody mediated immunological reactions. Cooperative interactions between T cells, macrophages(cells with a highly phagocytic capacity) and/or B cells are required for most immunological reactions to occur(55).

Differences in Purine Metabolism in Normal Lymphoid Cells

In cells from immunologically different lymphoid subpopulations, as well as in cells from subpopulations in various differentiation stages, differences in purine metabolism have been described. Differences in ADA activity between mature T and B lymphocytes do not seem to be significant(chapter 5, 124,174,183). Only one report(123) describes a significantly higher(40%) activity of ADA in T than in B cells. Although the relative activities of the T and B cell fractions in this report are in the same range as in other reports, in the latter this difference is not considered significant.

PNP activity has been suggested as a histochemical marker for

T cells in man(15). However, the data on this enzyme are rather confusing. Whereas in one report the higher PNP activity in T cells is confirmed(123), we could not find a significant difference between T and B lymphocytes(chapter 5, 183).

Many reports have been dealing with differences of 5'NT activity in T and B lymphocytes. It is generally accepted that ecto-5'NT activity is higher in human peripheral blood B cells than in T cells(chapter 5, 53,142,169,183). The relative differences however, vary from a moderately (53) to even a 15-fold(169) higher activity in B cells. The activity of ecto-5'NT appeared to be age dependent both in T and in B cells(17). From 40 years of age on, 5'NT activity decreases in peripheral blood T cells. In B cells a decreased ecto-5'NT activity was only seen at ages of 60 years or higher. To our knowledge no data have been reported on differences in AK activity between T and B cells.

Relatively little is known about differences in purine enzyme activities in T cell subfractions. We found that APRT, PNP and AK activities are significantly higher in T-Tγ peripheral blood cells than in Tγ cells(cells with a receptor for the Fc portion of IgG; chapter 5, 183). In contrast, a higher ADA activity was found in Tγ cells. Although in our studies this difference had no statistical significance, it should be mentioned that comparable results were reported by another group(159). Tγ cells have lower 5'NT activity than T-Tγ cells(chapter 5, 17,183). It was postulated that this difference in 5'NT together with the relative increase in the number of Tγ cells with age, explains the relatively low 5'NT activity in senescence(17).

According to the hypothesis that a relatively low 5'NT/kinase activity ratio causes cells to be more vulnerable towards deoxynucleoside toxicity(paragraph 2), one can reason that T cells are more vulnerable than B cells and that Tγ cells can be affected more easily than T-Tγ cells. However, especially concerning the last comparison more data have to be collected.

Not only peripheral blood lymphocytes exhibiting different phenotypes, but also lymphocytes from various lymphoid organs have been studied. Two major reasons could be given to study purine metabolism

in different lymphoid organs:

1. To know the possible variation of purine enzymes in different stages of lymphoid development may contribute to a better understanding of the role of purine metabolism in normal lymphoid differentiation. Not only differences between various subsets of PBL, but also differences between lymphocytes at various differentiation stages may cause selective sensitivity for purine nucleosides or their analogues.
2. If differences were found, this might help to a better understanding of the relation between purine metabolism and immune function.

Purine Metabolism in Different Mammalian Tissues

ADA activity, measured in several human tissues, was found to be high in thymus(chapter 9, 1,26,87,184). Intermediate ADA activities were found in human spleen(26,87) and peripheral blood lymphocytes (chapter 9, 31,87,184) and lymph node(1,87). Brain, kidney, liver and lung were found to have relatively low ADA activities(26,87). Considerable activities of several deoxynucleoside kinases were predominantly found in lymphoid tissues, where the thymus seemed to have the highest activity(26,40).

In contrast to the high activities of ADA and several deoxynucleoside kinases found in thymus tissue, a reletively low activity of 5'NT was seen(chapter 9, 40,53,184). The 5'NT activity in thymocytes could be increased by culturing them in medium containing thymic epithelial cell derived factors(41). As could be expected, the different enzymatic make-up of thymocytes as compared to PBL, leads to a different sensitivity towards deoxynucleosides. When culturing both thymocytes and PBL with deoxyguanosine, the proliferative response was affected mostly in the thymocytes(40). It was assumed that this phenomenon was due to the relativily poor ability of thymocytes to breakdown the accumulated deoxyGTP.

Our findings were not completely in agreement with these data (chapter 9). This might be ascribed to the different culturing conditions used. In our experiments thymocytes were cultured in the presence of thymic epithelial derived factor. Such factors are capable of

inducing 5'NT activity in thymocytes(chapter 9, 41), thus providing these cells with a better possibility to breakdown toxic deoxyribonucleotides. In experiments with deoxyadenosine comparable data were obtained(chapter 9, 184). Experiments carried out in thymocyte subfractions showed a good correlation between the ecto-5'NT/deoxycitidine kinase activity ratio and deoxynucleoside induced inhibition of PHA response(chapter 9, 184). Thymocytes were separated on size, with a centrifugal elutriation technique. It appeared that the fractions containing predominantly small sized cells were relatively immature. The fractions containing predominantly medium sized cells were found to be relatively mature(149). The thymocyte fraction with the highest ecto-5'NT/deoxycytidine activity ratio was affected least after culturing in the presence of deoxynucleosides, whereas the fraction with the lowest ratio was most seriously affected.

Besides differences in ecto-5'NT and deoxycytidine kinase activity, also differences in ADA and PNP activity were detected at the intrathymic level. Using an immunohistochemical technique, Chechik et al.(31) have shown that ADA positive cells were predominantly seen in the cortical regions of the human thymus. In the thymic medulla, ADA reactivity was observed only occasionally. It has been shown that the thymic cortex contains less mature lymphocytes than the medulla(chapter 9, 20). In the thymocyte fractions described above, the ADA/PNP activity ratio was highest in the relatively immature fractions and relatively low in the mature fractions(chapter 9, 184). From our experiments and ADA and PNP measurements in different rat lymphoid organs, where similar data were obtained(chapter 9, 11,12), it was suggested that the ADA/PNP activity ratio is a marker for T cell development. Deficiencies of each of these enzymes might affect T cells at separate stages of differentiation.

Although the physiological consequences of deficiencies of some purine enzymes have been extensively studied(paragraphs 1 and 2), the physiological role of purine metabolism in immune function is not yet fully understood.

The mechanisms which can lead to eventual cytotoxicity have extensively been discussed above(paragraph 2). From a number of in vitro and in vivo studies(177) it becomes more and more evident that purine analogues, metabolized especially by ADA and PNP, can act as regulators of several immune functions.

cAMP seems to be a good candidate for modulating the immune response. It is known that cAMP can stimulate both lymphocyte transformation and antibody secretion(83,84,101,161). Similar effects were seen with low concentrations of adenosine(150,180,183). These functions are inhibited by high concentrations of adenosine(chapter 10, 180). This is compatible with the finding that increased antibody production is related to increased ADA activity(71).

Astaldi et al.(9) have performed some interesting studies on a serum factor, which appeared to be adenosine. It was shown that this serum factor(adenosine), which could not be derived from thymectomized myasthenia gravis patients, was mainly active on the thymocytes(5,8). Among thymocytes the activity of the serum factor(adenosine) was found to be restricted to hydrocortisone sensitive(relatively immature) thymocytes(6). In vitro it was found that the serum factor increased cAMP levels in thymocytes(7). Protein synthesis was found to increase in thymocytes 15 min. after the serum factor(adenosine) was added(189). It was suggested that adenosine binds to thymocytes and then stimulates adenylate cyclase with a subsequent rise in intracellular cAMP. It is likely that this leads to an activation of protein kinases, which are responsible for the phosphorylation of proteins.

In relation to the sensitivity for theophylline(an inhibitor of phosphodiesterase, the enzyme that breaks down cAMP), different T cell subsets have been described(108). Those cells whose ability to bind sheep eryhtrocytes is not affected by theophylline treatment, are termed theophylline resistant(T_r), while those cells that lose the ability to form rosettes with sheep erythrocytes are termed theophylline sensitive (T_s). The T_r fraction contained mainly $T\gamma$ cells, whereas the T_s subset predominantly contained $T\mu$ cells(154). When adenosine is incubated with

peripheral blood T cells, an increase of the percentage of T_γ cells is seen(13). Adenosine however, not only increases the proportion of T_γ cells but also causes a loss of helper activity(13).

Comparable results were obtained with deoxyadenosine. In experiments where the number of plasma cells was counted after 7 days of culturing of PBL, in the presence of PWM a decrease of the number of plasma cells induced was significantly correlated with the concentration of deoxyadenosine added(73). It was concluded that deoxyadenosine, under the conditions used, affected the T helper cells. At very low deoxyadenosine concentrations however, the T helper function seems to be less affected than the T suppressor function(chapter 10, 180). Adenosine also seems to affect the T_μ cell subset(bearing the receptor for the Fc part of IgM). It was described(62,151) that this T cell subset also contains the precursors of the effector cells for cell mediated lympholysis. Wolberg et al. showed that adenosine inhibited the lymphocyte mediated lysis of tumor cells(187). Using a plaque forming cell assay, it was shown that deoxyguanosine at concentrations above 1 μM abolished T suppressor activity(64).

Apart of the studies mentioned in other paragraphs of this paper, some other data have to be mentioned about the relation of ADA and immune function. As could be expected from the foregoing, ADA plays an important role in T cell maturation. Incubation of precursor T cells from ADA deficient patients with thymic epithelial monolayers or thymosin, induced receptors for sheep erythrocytes(143,155). This induction could be prevented by adding the ADA inhibitor EHNA to the cultures (155). This indicated that, in particular at early stages of T cell maturation, the metabolic action of ADA is of importance. These results are compatible with data reported earlier(10,129). After 5 days of culturing, tonsil derived lymphoid precursor cells, inable to form rosettes with sheep erythrocytes, developed the capacity to form E-rosettes. When coformycin was added to the culture, no significant rosette forming capacity was found, even at 10 days of culturing(10). Addition of ADA to lymphocyte cultures of an ADA deficient SCID patient, restored the proliferative capacity of these cells(129).

Thuillier et al.(171) showed that immunocompetent rat thymocytes

were able to produce a T cell growth factor(TCGF) upon mitogen stimulation, but were not able to do so under ADA deficient conditions. Immuno-incompetent rat thymocytes differentiate and maturate in response to concanavalin A(ConA) if TCGF is added. This process can be inhibited by adding deoxycoformycin and deoxyadenosine. Through its catabolic activity, adenosine deaminase has been proposed as an enzyme controlling the amount of substrate available to xanthine oxidase(scheme 1). The latter enzyme produces O_2 radicals accompanying plasma membrane perturbation during phagocytosis of polymorphonuclear leukocytes(172). This illustrates that, when studying nucleoside toxicity towards the immune system, not only lymphoid cells should be considered.

The above data strongly indicate that normal immune function is strongly related to a properly functioning purine metabolism. These studies reported indicate that purine nucleosides not only affect the differentiation of T cells, but also modulate the functional activity of these cells. Adenosine, deoxyadenosine and deoxyguanosine may be considered as immunosuppresive and immunostimulatory agents, depending of the concentrations used, with a certain selectivity in their action.

4. BIOCHEMICAL PHENOTYPES OF CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA(ALL)

Patients with ALL can be classified by means of immunological markers. ALL patients with bone marrow that contains predominantly malignant cells bearing sIg, are referred to as B-ALL(table 2). When the lymphoblasts are predominantly forming E-rosettes and/or are positive with the anti-T cell antiserum, the patients are classified in the T-ALL group(68). The major subclass of ALL is the nonBnonT-ALL group (table 2), which can be discriminated from B-ALL by the absence of immunoglobulins on the lymphoblast membranes, and from T-ALL by the absence of E-rosetting capacity or reactivity with anti-T cell antiserum (57,68). However, patients who apparently had a considerable number of anti-T cell antiserum positive cells, have also been classified in the nonBnonT-ALL group, because no E-rosettes could be detected and the number of cells positive for the cALL(ALL with the major or common phenotype) antiserum(24,67), was predominant(34,68,91,181). The cALL antiserum provides a marker for further division of the nonBnonT-ALL

Table 2: Immunological Classification of Acute Lymphoblastic Leukemia

Classification	Relative Incidence	Markers
nonBnonT-ALL	± 80%	E^-, sIg^-, $anti\text{-}T^-$
unc.-ALL	± 15%	E^-, sIg^-, $anti\text{-}T^-$, $cALL^-$
cALL	± 65%	E^-, sIg^-, $anti\text{-}T^-$, $cALL^+$
preB-ALL	± 20%	E^-, sIg^-, $anti\text{-}T^-$, $cALL^+$, $cIgM^+$
T-ALL	± 20%	E^+, sIg^-, $anti\text{-}T^+$, $cALL^-$
B-ALL	± 2%	E^-, sIg^+, $anti\text{-}T^-$, $cALL^-$

cALL patients may occasionally have an $anti\text{-}T^+$ phenotype, whereas some T-ALL patients may occasionally have an E^- phenotype. E=rosette forming capacity with sheep erythrocytes. sIg=surface immunoglobulins. anti-T= anti-T cell antiserum. cIgM=cytoplasmic IgM heavy chains. cALL=common-ALL antiserum.

group. NonBnonT-ALL patients with leukemic cells positive for this antiserum are referred to as cALL(table 2), whereas the remaining patients, cALL negative, E-rosette negative, anti-T cell antiserum negative and sIg negative, are referred to as unclassified-ALL(unc.-ALL, table 2). The presence of cytoplasmic IgM heavy chains(cIgM) in the absence of sIg can be considered as a preB cell marker(63,135). Thus, nonBnonT-ALL patients with cells containing cIgM can be considered to be of the preB-ALL type(23,181).

In table 2 the classification of ALL patients is summarized. It appears that about 80% of the ALL patients cannot be classified as T- or B-ALL. From this nonBnonT-ALL group, about 65% of the patients can be classified as cALL, whereas about 15% remains unclassified(unc.-ALL, 68). From the cALL patients about 20% appeared to have cIgM and could be classified as preB-ALL(185). The phenotypes T-ALL and B-ALL were observed in about 15% and 2% of the ALL patients(68).

Although the data reported are not completely unanimous, a rather consistent picture of the biochemical phenotypes of different subclasses of ALL is emerging. Elevated levels of ADA in ALL lymphoblasts, as compared to control PBL, have been described in childhood ALL(chapter 6 and 7, 42,112,139,163,179,181). Conflicting findings by others(104, 159,191) can be explained by the fact that the group of patients was not completely in the childhood range(109,159) or that the patients

were not newly diagnosed, but were in relapse or in remission(191). In our studies it has been shown that this might affect purine enzyme activities(chapter 7, 181). The highest ADA activities were found in T-ALL, whereas nonBnonT-ALL patients had ADA activities intermediate between control PBL and T-ALL lymphoblasts(chapter 6, 42,139,163,179). generally an ADA activity in the range of control PBL has been found in B-ALL lymphoblasts(chapter 7, 42,181).

Differences in PNP activities in the various subgroups of ALL were not as pronounced as differences in ADA activity. In nonBnonT-ALL PNP activity levels were in the range of control PBL. In T-ALL however, a markedly decreased PNP activity was found(chapter 6, 14,179). The data on PNP activities in B-ALL patients are rather confusing. Patients with decreased(chapter 7, 139,181) as well as with increased PNP activity(chapter 7, 181) have been described. These different findings may indicate a heterogeneity in the leukemic cells isolated from B-ALL patients.

An inverse linear relationship has been found with T lymphoblasts, isolated from patients with T-ALL, between the number of E-rosette forming cells and the 5'NT activity(138). The 5'NT activity in T-ALL was significantly lower than in nonBnonT-ALL or in control PBL(chapter 6, 102,138,139,179). The mean 5'NT activity in nonBnonT-ALL seemed to be elevated as compared to control PBL(chapter 6, 102,179). Kramers et al.(102) described a mean 5'NT activity of $2.9 \pm 1.5\ 10^{-9}$ moles/mg protein.min. in normal lymphocytes. In nonBnonT-ALL cells a mean 5'NT activity of $5.2 \pm 2.0\ 10^{-9}$ moles/mg protein.min. was found. In comparable experiments described earlier(chapter 6, 179), we found mean 5'NT activities of 18.4 ± 11.7 and $25.9 \pm 23.1\ 10^{-9}$ moles/10^6 cells.hour in control PBL and nonBnonT leukemic cells, respectively. Due to the huge standard deviations, these differences were not found to be significant. When analyzing the cells of a number of nonBnonT-ALL patients in more detail, we found a possible explanation for this phenomenon. NonBnonT-ALL patients with cIgM appeared to have a significantly higher 5'NT activity than patients not having this preB cell marker(chapter 7,181). The number of patients who were investigated in this respect was rather limited. Nevertheless, when separating the group of nonBnonT-ALL patients in those with and those without cIgM in their cells, it

appeared that the first group(only 3 out of 7 patients) is responsible for the rather wide standard deviation and elevated mean 5'NT activity in the nonBnonT-ALL group. Considering the data described above, 5'NT activity cannot be a diagnostic marker for classification of subgroups of ALL.

In a systematic analysis of purine metabolizing enzymes in ALL, described in chapter 6(179), also differences in subgroups of ALL with respect to other enzymes became apparent. In nonBnonT-ALL significantly higher activities of HGPRT and adenylate kinase(AdKin) were found as compared to these activities in control PBL. APRT activity on the other hand was found to be lower in nonBnonT-ALL. Since HGPRT is catalyzing the supply of nucleotides via the purine salvage pathway and AdKin regulates the conversions of adenine nucleotides, these findings seem to be in agreement with the elevated ATP concentrations found in nonBnonT-ALL cells(47). In T-ALL, where the ATP concentrations were not significantly elevated, HGPRT was only moderately higher and AdKin showed activities comparable to control PBL(chapter 6, 179). APRT activity in T-ALL was about half the mean activity of control PBL.

When speculating on the biochemical differences caused by the different enzymatic make-up in nonBnonT-ALL and T-ALL, one might postulate that 5'NT is a key enzyme in this respect. Since 5'NT activity in nonBnonT-ALL seems to be normal to somewhat elevated, breakdown of adenine and other purine nucleotides can occur at a normal rate. Since ADA activity is higher, the catabolic action of 5'NT might be followed by rapid deamination of adenosine to inosine. Since there is no reason to assume that the apparently normal PNP activity is rate limiting in any way, the consequence of this might be a more extensive production of hypoxanthine and subsequently a more extensive loss of the purine base in uric acid. However, the cell has two rescue mechanisms. The first is the elevated capacity of HGPRT to salvage hypoxanthine formed, and recycle it into the nucleotide pool. The second is an elevated AdKin activity which helps the cell to protect the adenine nucleotides, formed from either IMP or the action of AK(scheme 1), from dephosphorylation by 5'NT more effectively. This is demonstrated by the elevated ATP pools in nonBnonT leukemic cells.

In the case of T-ALL, the very high ADA activity might be relatively unimportant, since both the relatively low 5'NT and PNP activity might be rate limiting in the catabolism of adenine nucleotides. In T-ALL the malignant lymphoblasts represent relatively early stages of T cell differentiation(chapter 9). Apparently a low nucleotide breakdown and a high adenosine catabolism is necessary for differentiating T cells. In this case the cell apparently does not need to have either high HGPRT or AdKin activity as is the case in nonBnonT-ALL.

5. BIOCHEMICAL PHENOTYPES OF CHRONIC LYMPHOCYTIC LEUKEMIA(CLL)

In B-CLL patients markedly altered activities of ADA, PNP and 5'NT have been described. ADA activity in several patients with CLL was found to be considerably lower than in control PBL(chapter 8, 3,90,112,137, 174,182) In a number of CLL patients ADA activity was in the range of the control value(chapter 8, 162,181). This apparently wide activity range in patients with CLL becomes also evident from the finding that B-CLL patients with paraproteinaemia had a significantly lower ADA activity than patients without paraproteinaemia(chapter 8, 182).

Mean PNP activity was found to be relatively low in patients with CLL(chapter 8, 3,16,182). A comparable wide activity range was found with respect to PNP activity in CLL as was the case with ADA. However, no differences between CLL patients with and without paraproteinaemia were noted(chapter 8, 182). 5'NT activity was found to be low in CLL patients(chapter 8, 102,110,134,160,182). However, a clear distinction between the CLL patients with different phenotypes must still be made. 5'NT activity was only found to be low in B-CLL. In two patients with T-CLL a 5'NT activity comparable to the level found in control PBL was observed(102). In the B-CLL patients a considerable range in 5'NT activities was seen(chapter 8, 110,182). In B-CLL with paraproteinaemia 5'NT activity was 11.5 10^{-9} moles/10^{6} cells.hour, whereas patients without paraproteinaemia had a mean 5'NT level of 1.2 10^{-9} moles/10^{6} cells.hour(chapter 8, 182).

Significantly elevated activity levels of enzymes involved in adenine nucleotide metabolism(AK and AdKin) have been found(chapter 8,

182). Whether these different enzyme activities have any consequences for the nucleotide pools in B-CLL lymphoblasts, remains to be investigated.

6. RELATIONS BETWEEN LYMPHOBLASTIC LEUKEMIA AND NORMAL LYMPHOID CELL DIFFERENTIATION

The immunological markers typical for different subgroups of leukemia, are believed to reflect the stage of differentiation of the malignant clone(68). Thus, in principle, in should be possible to "map" the several subgroups of human lymphoid malignancies along the different lymphoid differentiation lines. Knowledge of the enzymatic make-up in various differentiation stages might contribute to a better understanding of the role of purine metabolism during normal lymphoid differentiation and in leukemia.

Greaves and Janossy(68) proposed in their "phenotypic map of human malignancies" that the nonBnonT-ALL group represented a common stage in early B and T cell lineage. Indeed there is evidence that the so called nonBnonT-ALL(neither sIg nor E-rosette positive, table 2) is a heterogeneous group of leukemias. A number of leukemias with undetectable conventional T and B cell markers appeared to have cIgM(chapter 7, 181, 185) a preB cell marker(63). The cells of the latter patients were positive with the cALL antiserum. Stimulation of bone marrow cells from patients with nonBnonT-ALL with thymic factors, induced immuno-competent T cells as detected by a local Graft versus Host reaction(152,153). Chiao et al.(34) cultured cells lacking B and T lymphocyte markers from peripheral blood of two ALL patients in the presence of medium produced from PHA stimulated allogeneic lymphocytes. Within 18 hours these cells acquired the capacity to form rosettes with sheep erythrocytes and to bind IgM or IgG. These data support the view that the nonBnonT cell compartment of peripheral blood mononuclear cells appears to contain precursor cells capable of generating the entire spectrum of lymphopoietic cells(32). As Greaves and Janossy stated: "some of the nonBnonT or common ALL's are preB, some preT and some may be 'frozen' at the level of a common(T and B) lymphoid precursor cell or a pluripotent stem cell"(68).

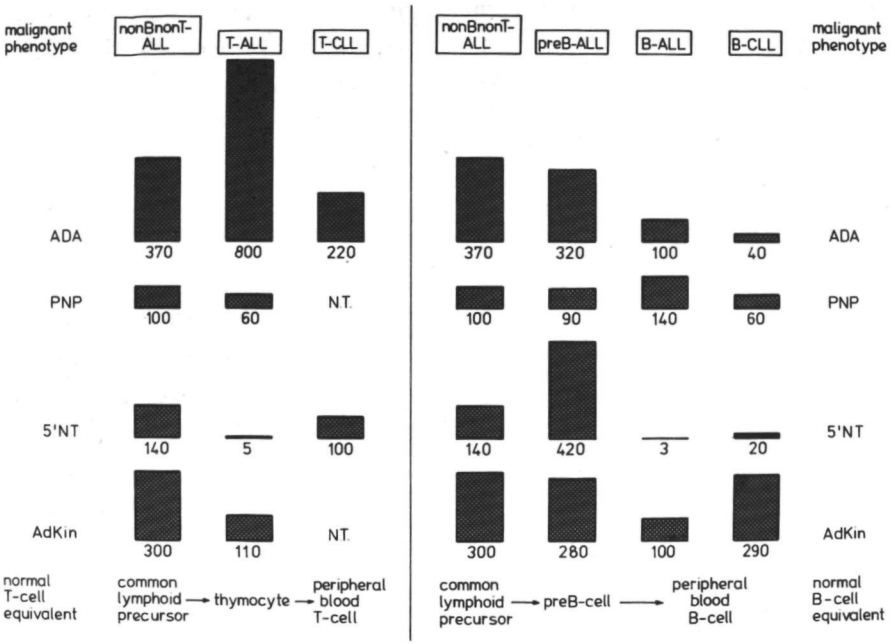

Figure 4: The relative enzyme activities in different subtypes of human leukemia as compared to the enzyme activities in control peripheral blood lymphocytes(=100 arbitrary units) is indicated. The relative enzyme activities from nonBnonT-ALL(chapter 6, 179), T-ALL (chapter 6, 179), T-CLL(102), preB-ALL(chapter 7, 181), B-ALL(chapter 7, 181) and B-CLL(chapter 8, 182) are extrapolated from data obtained mostly by our own group, which are reported elsewhere. NT=not tested.

As the nonBnonT-ALL can be considered as the malignant equivalent of a common lymphoid precursor cell, T-ALL can be considered as the malignant counterpart of a stage in the T cell lineage(68). This type of ALL could more or less be a phenotypical equivalent to the thymocytes. The mature T cell has its equivalent in the T-CLL subgroup(68).

As in the T cell lineage, several stages of the B cell lineage have their maligant counterparts. The preB ALL represents the preB cell differentiation stage, the mature B cells are represented by B-ALL and B-CLL(68). Myeloma cells are the malignant equivalent of actively Ig secreting B lymphocytes or plasma cells.

Taking the peripheral blood cells as reference, a hypothesis for the enzymatic changes in human lymphoid differentiation based on the

data described above can be proposed(fig. 4).

In nonBnonT-ALL a relatively high ADA activity is observed. T-ALL cells have ADA activities comparable to those in thymocytes, whereas in T-CLL, ADA activity was found to be in the range of control peripheral T cells(174). As depicted in fig 4., ADA activity is intermediate at the level of the lymphoid precursor cell, high at the preT/thymocyte level and relatively low in the more mature T cell. In the B cell lineage the ADA activity seems to be maximal in the very early differentiation stages(cf. nonBnonT/preB-ALL). In the more mature differentiation stages (cf. B-ALL and B-CLL) mean ADA activity is relatively low.

The changes in PNP activity in this model of lymphoid cell differentiation are not as pronounced as is the case with ADA. Both in B and T cell lineage the PNP activity levels of the common lymphoid stem cell compartment(cf. nonBnonT-ALL) are comparable to the activities in peripheral B and T cells. The intermediate stages of T cell differentiation seem to have a moderately lower(cf. T-ALL) PNP activity. In B-CLL the PNP activity is slightly decreased.

In relatively early(cf. nonBnonT-ALL) and relatively late(cf. T-CLL and PBL-T) stages of differentiation in the T cell lineage, comparable 5'NT activities were found. Intermediate stages of the T lineage seem to be characterized by low, if any, 5'NT activity(chapter 9, 184). The situation in the B cell lineage model seems to be rather different (fig. 4). Not only higher activities of 5'NT in peripheral B lymphocytes than in T lymphocytes were seen, but in contrast with preT cells, in preB cells a relatively high 5'NT activity was observed(chapter 7, 181).

AdKin activity seemed to be highest in the common B and T cell precursor cells(cf. nonBnonT-ALL). The stages of T cell differentiation comparable with T-ALL were found to have relatively low AdKin activities that were not changed significantly. An increased AdKin activity in B cell lineage apparently occurred at all differentiation stages shown (fig. 4), except for the stage that was related to B-ALL.

The schematic representation of B and T cell lineage should be

considered as very provisional. The leukemias are depicted in positions equivalent to normal cells to which they appear to have the closest phenotypic resemblance(68). When studies are performed in more detail, it might become clear that, for instance T-ALL and thymocytes or B-CLL and B-ALL should be shifted somewhat along their respective lineages. Also with regard to T-CLL and the B-ALL subclasses, more patients should be investigated in order to establish a more realistic scheme.

However, these points probably will not significantly alter the general conclusions, that

1. T-cells at various stages of differentiation have higher ADA activities than differentiating B cells.
2. PNP activities only seem to drop in preT cell stages.
3. As compared to mature T cells, preT cells are characterized by high ADA, low PNP, low 5'NT and essentially normal AdKin activities.
4. As compared to mature B cells, preB cells are characterized by intermediate to low ADA, normal PNP, high 5'NT and high AdKin activities.

Unfortunately, since the normal counterparts of these malignancies are not easily available, it is only possible to test these conclusions on a few points. From the foregoing it must be clear that not only the immunological phenotype of T-ALL cells is alike that of thymocytes, but also the biochemical characteristics have much in common. T lymphoblasts as well as thymocytes can be characterized by high ADA, low 5'NT and relatively low PNP activity. From the enzyme activities found in T-CLL and in B-CLL/B-ALL it might be concluded that the normal counterparts are indeed situated quite near the mature T and B cells in their respective lineages. The enzyme activities of PNP, ADA and 5'NT in T-CLL and B-CLL/B-ALL only show minor differences with the activities found in T and B cells, respectively. As suggested before, B-CLL and B-ALL might have to change places along the B cell axis. However, with regard to one enzyme activity(AdKin), they clearly seem to have more in common with the preB phenotype than with the mature B cell.

The expression of purine enzyme activities during the differentiation of lymphoid cells deserves to be studied in detail. Then enzymes of purine metabolism cannot only serve as diagnostical markers in various lymphoid malignancies, but since a different enzymatic make-up

of purine metabolism might cause different sensitivities towards several purine analogues, this might also eventually lead to a chemotherapy specifically directed towards the (malignant) cells in a certain stage of lymphoid differentiation. If this approach appears to be successful, certain types of leukemia might be treated with less side effects on the normal lymphoid population and other tissues.

7. NUCLEOSIDE TOXICITY AND POSSIBILITIES FOR ENZYME DIRECTED CHEMOTHERAPY

The point of view that chemotherapy with purine analogues can be directed against certain cells with a specific purine enzymatic make-up is supported by some experimental evidence. Some cells seem to be resistent against the broadly used anti-cancer agent and purine derivative 6-mercaptopurine(6MP). In a review(22) Brockman argued that not only bacteria, but also neoplastic cells resistant to 6MP and 8-azaguanine lacked the capacity to form nucleotides of these analogues. This failure seemed to be a consequence of a marked decrease or loss of HGPRT activity. It was suggested that loss of HGPRT activity in human leukemia cells should be recognized as a clinical resistance mechanism (21,22,46). Alternative explanations for resistence to purine analogues were among others: decrease or loss of kinase activity which prevents cells to form eventually toxic nucleotides and increased degrading capacity of the purine analogues to 6-thioxanthine or 6-hydroxypurines (22,75).

Extrapolating these findings to leukemia, this might mean that leukemic cells having relatively high HGPRT(nucleotide synthesis, scheme 1) and low 5'NT(nucleotide breakdown) are affected more than normal cells with lower HGPRT and higher 5'NT activities. These enzymatic properties can be found in T-ALL lymphoblasts(chapter 6, 179). When these cells would be treated with 6MP, the formation of the toxic intermediate 6-thio-IMP pool can be expected to be more extended than in normal lymphocytes or preB leukemic cells that can easily breakdown (normal or high 5'NT activity, scheme 1) this nucleotide(chapter 7, 181). Moreover in T-ALL blasts the side-way conversion of 6MP to 6-thioinosine by the action of PNP would be rather limited.

Not only enzyme activities are capable of influencing specific chemotherapeutic sensitivity in human lymphoid cells. Also altered kinetic properties may play a role in this respect. Jackson et al.(94) demonstrated that cultured lymphoid cells with a decreased affinity of dihydrofolate reductase for methotrexate(MTX) is accompanied by resistance to the drug. Similar alterations in kinetic properties of dihydrofolate reductase have been seen in human lymphoblastoid lines resistant to MTX(95).

Resistance towards 5-fluorouracil(5FU) was seen(74) in cells where thymidilate synthetase has lost the binding capacity of 5-fluoro-deoxy-UMP(5FdUMP). 5FdUMP is the activated inhibitor of 5FU which exerts a strong feed-back inhibition of thymidilate synthesis(45). Examples like these emphasize the need, not only for systematic analysis of purine and pyrimidine interconversions in malignant cells, but also for kinetic studies with respect to the affinity of antimetabolites or their derivatives towards their targets.

Perhaps even more important than the chemotherapeutic actions of synthetic drugs, the inhibitory effects of accumulated naturally occurring purine and pyrimidine nucleosides may be considered. The differential sensitivity of T and B cells towards purine nucleosides has been discussed in another part of this paper(paragraph 2). Fox et al.(61) have shown that some nonBnonT leukemic cell lines exhibited a sensitivity towards deoxyguanosine, deoxyadenosine, deoxycytidine and thymidine comparable to the sensitivity shown in some leukemic T cell lines. According to these authors, the sensitivity of T and nonBnonT cells and the relative non-sensitivity of B cells could not be ascribed to a difference in ecto-5'NT activity as postulated before. NonBnonT leukemic cell lines were found to have ecto-5'NT activities significantly higher than T leukemic cell lines and in the range of Epstein-Barr-virus(EBV)-transformed B cell lines(60). Recently, in a report from the same group, it was shown that T and nonBnonT cell lines were essentially deficient of ecto adenosinetriphosphatase(ecto-ATPase) activity, whereas B cell lines displayed a considerable activity of this enzyme. As this cell surface enzyme seems to have a broad substrate specificity and deoxyATP and deoxyGTP are believed to be the major toxic metabolites, low ecto-ATPase activity may represent a mechanism for increased sensitivity to

deoxynucleoside growth inhibition(89). The many examples of the apparently enzymatically regulated cytotoxicity of nucleosides, make these compounds appropriate candidates for developing lymphoid cell subtype specific chemotherapy based on the enzymatic make-up of the present subtype.

Based on studies on the biochemical mechanisms leading to a combined immune defect in ADA deficiency and the enzymatic differences between leukemic and normal lymphoid cells, an alternative approach to anti-leukemia chemotherapy was explored. Prentice and his group were able to induce a considerable decrease in lymphoblasts in bone marrow of two patients with T-ALL, who were resistant to conventional forms of chemotherapy. These authors(132) were able to induce an almost complete remission of leukemic blasts, using the ADA inhibitor deoxycoformycin (dCF). The dCF treatment was accompanied by a severe lymphopenia and the patients died of a progressive measles infection and leukemic meningitis, respectively. In subsequent more extensive studies, dCF was administered to ALL patients irrespective of the subtype of the leukemias (131,164). In these studies also a severe lymphopenia was noted. In most of the patients no decrease in neutrophils, platelets and Hb in peripheral blood were seen after 1 or more moderate courses of dCF(164). Only after 5 courses of 0.25 mg dCF/kg body weight, moderate decreases of these parameters were noted(164). In the 7 patients studied by Smyth et al.(164) three partial responses were seen. In the studies performed by Poplack et al.(131) dCF exerted an anti-tumor activity in 8 out of 26 ALL patients, two of whome experienced a complete remission.

The above data illustrate that the success of purine enzyme directed chemotherapy might be related to the enzymatic make-up of the leukemic cells. Since nonBnonT and preB leukemias have normal to elevated activities of 5'NT, the accumulation of toxic deoxynucleotides from adenosine can relatively easily be overcome by the breakdown of these compounds by 5'NT. It can be surmized that the heterogeneous reactions to dCF in the patients in the two studies described above are due to the difference in enzymatic make-up of the subtypes of ALL.

A correlation of dCF induced remission and deoxyATP accumulation has been reported recently(133,144). Subtypes of ALL which, due to the

enzymatic make-up, could not be expected to accumulate deoxyATP, do not respond to dCF therapy. From the 12 patients with T-ALL, treated with dCF, 7 went into complete remission, 2 others showed a good partial response and 3 patients were resistant to dCF(133). Three patients showing a rapid in vitro accumulation of deoxyATP in the blast cells, achieved complete remission. Two patients, with no clinical response, showed poor deoxyATP accumulation. One case, having an intermediate pattern, had a good partial remission. When studying these patients in more detail, it appeared that the patients with complete remission accumulated deoxyATP relatively fast in the lymphoid cells. The deoxyATP accumulation was accompanied by a reduction in ADA activity and the lymphoid blast count. In a patient with partial remission these processes went relatively slow and in the poorly responding patients a decrease in blasts was seen rather late(144). The inactivation of SAHH activity was following the decrease in the number of leukemic blasts. Thus potentially decreased methylation pathways, as a consequence to lowered SAHH activities, seem of limited importance in the toxic actions as a consequence of nucleoside intoxication.

These data emphasize that a careful systematic analysis of adenine nucleotide metabolism and a selective use of dCF may contribute to the success of this approach. Although effects of dCF on other tissues(e.g. nervous system, 111) should be evaluated, it seems that "nucleoside intoxication" might be promising with respect to certain types of ALL.

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SCHEME 1: Simplified Scheme of Purine and Pyrimidine Metabolism

Enzymes:

1 - HGPRT, hypoxanthine-guanine phosphoribosyltransferase
2 - APRT, adenine phosphribosyltransferase
3 - ADA, adenosine deaminase
4 - PNP, purine nucleoside phosphorylase
5 - AK, adenosine kinase
6 - AdKin, adenylate kinase
7 - dCK, deoxycytidine kinase
8 - 5'NT, purine-5'nucleotidase
9 - AMPD, AMP deaminase
10 - OPRT, orotidine phosphoribosyltransferase
11 - ribonucleotide reductase
12 - XO, xanthine oxidase

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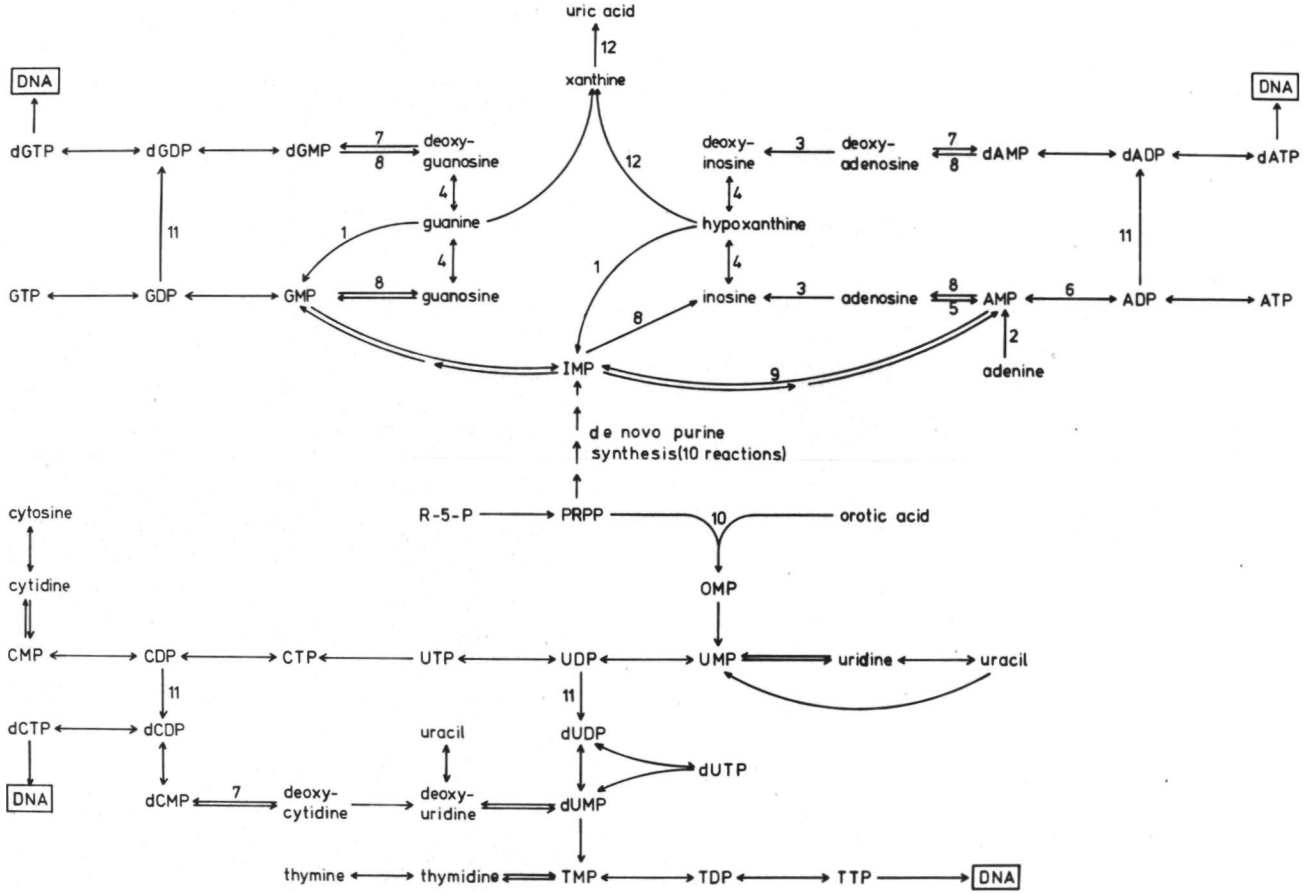
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xanthine
DNA
dGTP
dGDP
dGMP
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deoxy-
guanosine
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adenosine
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dAMP
dADP
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DNA
4
guanine
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hypoxanthine
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GTP
GDP
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adenosine
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ADP
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adenine
IMP
9
de novo purine
synthesis(10 reactions)
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R-5-P
PRPP
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After the intr ductory remarks(chapter 1), the developement of an enzymatic micromethod and its application in the enzymatic analysis of purine metabolism in lymphocytes and subpopulations is described in the section "Enzymological Analysis of Purine Metabolism in Lymphoid Cells". Lyophilization of the cells and subsequent resuspension of the freeze-dried material appeared to yield maximum enzyme activities(chapter 2). The method was improved by adding 0.67%(v/v) BSA to the cell suspensions (chapter 3). Purine enzyme activities in lymphocytes stimulated with PHA did not change significantly, whereas in PWM stimulated lymphocytes, contaminated with some monocytes, markedly increased HGPRT, ADA and 5'NT activities were observed(chapter 4). Differences in purine enzyme activities found in subpopulations of human peripheral blood lymphocytes are described in chapter 5. T cells have lower HGPRT, AK, 5'NT and AMPD activities than B cells. Differences in PNP and AdKin activities found in the subpopulations could, at least in part, be ascribed to platelet contamination of the B cell fraction. When comparing enzyme activities in lymphoid cell subfractions it is necessary to carefully characterize the various subfractions, in order to account for the different isolation procedures. T_γ cells had lower activities of APRT, PNP and AK than T-T_γ cells. It is suggested that T_γ cells might be more vulnerable to purine (deoxy)nucleoside intoxication than T-T_γ cells.

In the second section, the expression of purine metabolism in different lymphoid leukemia's is described. In chapter 6 evidence is presented that certain purine enzymes can be useful as markers for the subclassification of the two major ALL subclasses, T-ALL and nonBnonT-ALL. As compared to control PBL, cells from patients with nonBnonT-ALL have higher HGPRT, ADA and AdKin activities and a lower APRT activity. In T-ALL, HGPRT and ADA activities were also elevated as compared to control PBL, whereas APRT, PNP, 5'NT and AMPD activities were found to be lower. In chapter 7 several purine enzyme activities have been correlated with immunological markers of ALL patients. Relatively high ADA, low PNP and low 5'NT activities were associated with the presence of the E-rosette marker and the absence of the cALL marker. Intermediate ADA activities and high AdKin activities were associated with the presence of the cALL marker and the absence of the E-rosette marker. PreB-cALL

cells appeared to have about twice the 5'NT activity of cALL cells lacking the preB marker. The enzymatic make-up of malignant cells from two B-ALL patients differed markedly. 5'NT activity was found to be low in these patients. Therefore, 5'NT cannot serve as a biochemical marker for different subtypes of ALL. On the other hand, ADA and AdKin activity seem to be promising in this respect. The decrease in ADA activity in nonBnonT-ALL patients after prednisone monotherapy could not be correlated with a decrease in the number of proliferating cells. NonBnonT-ALL patients in relapse show 5'NT activities of about 30% of the 5'NT activities in leukemic cells from newly diagnosed nonBnonT-ALL patients. It is concluded that due to the specific enzymatic make-up several subgroups of ALL will display a different sensitivity towards purine (deoxy)-nucleosides. In lymphoblasts from CLL patients(chapter 8) low ADA, 5'NT and PNP activities and high AK and AdKin activities as compared to control PBL have been observed. CLL patients without paraproteinaemia had about twice as high an ADA activity and about 10 times as low a 5'NT activity as compared to patients with paraproteinaemia. A relation between 5'NT activity and Ig production is suggested. No correlations between purine enzyme activity levels and the E-rosette marker or the sIg marker were found. The percentage of cells rosetting with mouse erythrocytes however, seemed to correlate significantly with 5'NT activity.

Some aspects of nucleoside toxicity in mature and immature lymphoid cells are described in the third section. Thymocytes appeared to have higher ADA, lower PNP and lower ecto-5'NT activities than peripheral blood T cells. When separating thymocytes into six subfractions according to stage of maturation, it appeared that the ADA/PNP activity ratio was highest in fraction 1(containing the relatively most immature cells) and lowest in fraction 6(containing the relatively most mature thymocytes). It was concluded that the ADA/PNP activity ratio was a marker for intrathymic maturation. The ecto-5'NT/dCK activity ratio was found to be maximal in fraction 3. This activity ratio parallelled the inhibition of the PHA response by (deoxy)nucleosides. From the experiments described in chapter 10 it was concluded that T suppressor cells might be more vulnerable to (deoxy)nucleoside intoxication than T helper cells. In these experiments, where the mitogen induced proliferative response, protein synthesis and Ig secretion were assessed, it was

shown that deoxyadenosine at low concentration enhanced Ig secretion, whereas the proliferative response was inhibited.

In chapter 11 the relation between purine metabolism, leukemia and lymphoid cell differentiation is reviewed and considered in the context of the findings reported in this thesis.

Na enkele introducerende opmerkingen(hoofdstuk 1), is in de sectie "Enzymological Analysis of Purine Metabolism in Lymphoid Cells" de ontwikkeling van een enzymatische micromethode en de toepassing hiervan bij de enzymatische analyse van het purine metabolisme in lymphocyten en subpopulaties beschreven. Droogvriezen van de cellen en het vervolgens weer opnemen van het droog gevroren materiaal bleek maximale enzymactiviteiten te geven(hoofdstuk 2). De methode werd verbeterd door 0.67% (v/v) BSA aan de celsuspensie toe te voegen(hoofdstuk 3). Purine enzymactiviteiten in lymphocyten, gestimuleerd door PHA, veranderen niet of nauwelijks, terwijl in door PWM gestimuleerde lymphocyten, vermengd met enkele monocyten, aanmerkelijk verhoogde activiteiten van HGPRT, ADA en 5'NT werden gemeten(hoofdstuk 4). Verschillen in purine enzymactiviteiten, die gevonden zijn in subpopulaties van menselijke perifeer bloed lymphocyten, zijn beschreven in hoofdstuk 5. T cellen hebben een lagere activiteit van HGPRT, AK, 5'NT en AMPD dan B cellen. De verschillen in PNP en AdKin activiteit, die gevonden zijn in deze subpopulaties, konden, tenminste voor een deel, worden toegeschreven aan verontreiniging van de B cell fractie met bloedplaatjes. Als men de enzymactiviteiten in lymphoide cell subfracties vergelijkt, is het noodzakelijk om de verscheidene subfracties zorgvuldig the karakteriseren i.v.m. de verschillende isolatie procedures. T_γ cellen hebben een lagere activiteit van APRT, PNP en AK dan T-T_γ cellen. De suggestie wordt gedaan dat T_γ cellen kwetsbaarder zouden kunnen zijn voor purine (deoxy)nucleoside vergiftiging dan T-T_γ cellen.

In de tweede sectie wordt de expressie van het purinemetabolisme in verschillende lymphoide leukemieën beschreven. In hoofdstuk 6 worden gegevens gepresenteerd, die ondersteunen dat bepaalde purine enzymen bruikbaar kunnen zijn bij de subclassificatie van de twee voornaamste ALL subclassen, T-ALL en nonBnonT-ALL. Vergeleken met controle PBL hebben cellen van patienten met nonBnonT-ALL hogere HGPRT, ADA en AdKin activiteiten en een lagere APRT activiteit. In cellen van patienten met T-ALL werden de activiteiten van HGPRT en ADA ook verhoogd gevonden ten opzichte van controle PBL, terwijl de activiteiten van APRT, PNP, 5'NT en AMPD lager waren. In hoofdstuk 7 zijn verscheidene purine enzymactiviteiten gecorreleerd met immunologische kenmerken van ALL

patienten. Relatief hoge ADA, lage PNP en lage 5'NT activiteiten waren geassocieerd met het aanwezig zijn van het E-rozet kenmerk en de afwezigheid van cALL kenmerken. Intermediaire ADA activiteiten en hoge AdKin activiteiten werden geassocieerd met de aanwezigheid van het cALL phenotype en de afwezigheid van het E-rozet kenmerk. PreB-cALL cellen bleken ongeveer de dubbele 5'NT activiteit te hebben van cALL cellen die het preB kenmerk misten. De enzymatische "make-up" van maligne cellen van twee B-ALL patienten verschilde aanzienlijk. Onder andere werd een lage 5'NT activiteit gevonden bij deze patienten. Daarom kan 5'NT niet dienen als biochemische parameter voor de verschillende subtypen van ALL. Anderzijds schijnen ADA en AdKin in dit opzicht veel belovend te zijn. De verlaging van de ADA activiteit in patienten met nonBnonT-ALL na prednison monotherapie kon niet worden gecorreleerd met een verlaging van het aantal delende cellen. Patienten met nonBnonT-ALL in "relapse" hebben 5'NT activiteiten, die ongeveer 30% zijn van de 5'NT activiteiten in nieuw gediagnostiseerde patienten met nonBnonT-ALL. De conclusie wordt getrokken, dat door de specifieke enzymatische "make-up" verscheidene ALL subgroepen een verschillende gevoeligheid voor purine (deoxy)-nucleosiden zullen hebben. In lymphoblasten van patienten met CLL(hoofdstuk 8) zijn lage ADA, 5'NT en PNP activiteiten en hoge AK en AdKin activiteiten gevonden, vergeleken met de controle PBL. CLL patienten zonder paraproteinaemie hadden ongeveer een twee maal zo hoge ADA activiteit en een ongeveer 10 maal lagere 5'NT activiteit dan de patienten met paraproteinaemie. Een relatie tussen de 5'NT activiteit en Ig productie wordt gesuggereerd. Er zijn geen relaties gevonden tussen purine enzymactiviteiten en het E-rozet kenmerk. Het percentage cellen, dat rozetteert met muizen rode bloed cellen, scheen echter significant te correleren met de 5'NT activiteit.

Enkele aspecten van nucleoside intoxicatie in rijpe en onrijpe lymphoide cellen worden beschreven in de derde sectie. Thymocyten bleken een hogere ADA, lagere PNP en lagere ecto-5'NT activiteit te hebben dan perifeer bloed T cellen. Als thymocyten gescheiden werden in zes subfracties, naar het stadium van rijping, bleek dat de ADA/PNP activiteits ratio het hoogst was in fractie 1(de meeste onrijpe cellen) en het laagst in fractie 6(het meeste rijpe cellen). De conclusie was, dat de ADA/PNP activiteits ratio een kenmerk is voor de rijping in de thymus. De ecto-5'NT/dCK activiteits ratio was maximaal in thymocyten fractie 3.

Deze ratio liep parallel aan de remming van de PHA respons door (deoxy)-nucleosiden. Uit de experimenten beschreven in hoofdstuk 10 werd geconcludeerd, dat T suppressor cellen kwetsbaarder zouden kunnen zijn voor (deoxy)nucleosiden vergiftiging dan T helper cellen. In deze experimenten, waarin de mitogeen geinduceerde delingsrespons, eiwit-synthese en Ig secretie werden bepaald, werd aangetoond dat lage concentraties van deoxyadenosine de Ig secretie verhoogden, terwijl de delingsrespons geremd werd.

In hoofdstuk 11 wordt een overzicht gegeven van de relatie tussen purine metabolism. leukemie en lymphoide cell differentiatie en beschouwd in samenhang met de bevindingen beschreven in dit proefschrift.

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STELLINGEN

bij het proefschrift

STUDIES ON PURINE METABOLISM
IN RELATION TO LEUKEMIA
AND LYMPHOID CELL DIFFERENTIATION

I

De verandering in de ratio van de activiteit van adenosine deaminase en purine nucleoside phosphorylase is een goed kenmerk voor T cel differentiatie.

II

Het is niet geoorloofd om het effect van purine nucleosiden op de stimulatie van de totale thymocyten populatie door phytohaemagglutinine, te relateren aan de activiteit van purine nucleosiden metaboliserende enzymen.

III

Bij de interpretatie van verschillen in activiteiten van enzymen uit het purine metabolisme tussen lymphoide cel subpopulaties, speelt de methode van celscheiding een essentiële rol.

IV

Bij het ontwikkelen van chemotherapeutica dient meer dan voorheen aandacht geschonken te worden aan specifieke metabole eigenschappen van maligne cellen.

V

Recente ontwikkelingen bij de bestudering van o.a. hematologische afwijkingen, immuun deficiënties en spierklachten, wijzen op de noodzaak van screening van purine- en pyrimidine verbindingen bij een groot aantal patiënten.

VI

Het vermelden van de resultaten van de koude lymphocytotoxische antilichaam test zonder opgave van de isolatieprocedure van de targetcellen, is weinig zinvol

VII

Bij de diagnostiek van maligne lymphomen heeft onderzoek naar het immunologisch phenotype aan celsuspensies geisoleerd uit de lymfeklier een beperkte waarde.

VIII

De biochemische screening van patiënten met mentale retardatie is slechts van betrekkelijke waarde.

IX

Ook dragers van volledige gebits-prothesen moeten jaarlijks naar de tandarts.

X

Als medium om hoog radioactief afval op te slaan is "synrock" beter geschikt dan glas.

XI

De huidige consumptie van sojaproducten in de westerse wereld draagt niet bij tot het oplossen van het hongerprobleem in Latijns-Amerika.

XII

Beperken van de kosten van de gezondheidszorg is meer een kwestie van beheersing dan van bezuiniging.

XIII

Door inkomensmatiging en het daardoor toenemend aantal werkzoekenden zal de werkloosheid niet afnemen.

XIV

Het inzetten van de Chinese graskarper als slootreiniger dient achterwege te blijven.

XV

In de kaken van Homo sapiens is steeds minder plaats voor verstandskiezen.

XVI

In het kader van de kostenbeheersing in de gezondheidszorg dient de uitdrukking "de pil vergulden" uit het nederlands taalgebruik geschrapt te worden.

J.P.R.M. van Laarhoven 7 oktober 1982

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