STUDIES ON HUMAN LYMPHOCYTE 5'-NUCLEOTIDASE

by

MARTIN ROWE

Division of Immunological Medicine
Clinical Research Centre
Harrow

Registered with:
Bedford College
Regents Park
London

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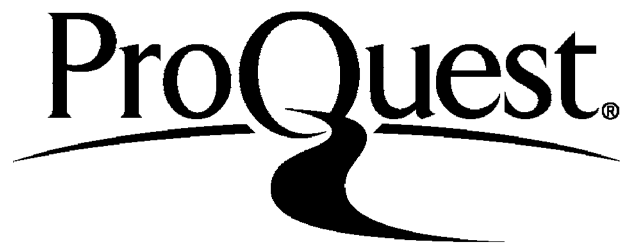

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ABSTRACT

The partial deficiency of 5'-nucleotidase activity found on the circulating mononuclear cells of many patients with 'common variable' or 'X-linked' hypogammaglobulinaemia was not due to abnormal compartmentalisation of the enzyme, altered enzyme kinetics, or the presence of a regulatory factor. Isolation of subpopulations of mononuclear cells from normal adult blood showed that B-lymphocytes have about four times more 5'-nucleotidase activity per cell than T-lymphocytes, and that monocytes have very little activity. Thus the absence of circulating B-lymphocytes in patients with X-linked hypogammaglobulinaemia contributed to the low activity in these patients, but some patients also had a low level of T-cell 5'-nucleotidase activity. The low enzyme level in 'common variable' hypogammaglobulinaemia was due to one or more of three factors: a low T-cell activity, a reduced percentage of B-cells, and a low activity on the B-cells.

The ability of circulating mononuclear cells to migrate out of capillary tube cultures *in vitro* was unimpaired, and inhibition of the 5'-nucleotidase activity of normal cells did not affect their migration. Inhibition of the 5'-nucleotidase activity also had no effect on the transformation or immunoglobulin production of tonsil lymphocytes *in vitro*. However, changes in the enzyme activity on tonsil lymphocytes were observed during cell transformation. Comparison of the 5'-nucleotidase activities of the circulating mononuclear cells from normal adults, cord blood, patients with chronic lymphatic leukemia, and from patients with hypogammaglobulinaemia has led to the hypothesis that the low level of the enzyme on the lymphocytes of patients with 'common variable' and 'X-linked' hypogammaglobulinaemia is not the cause of the disease but reflects a stage of maturation arrest in these cells.

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ABBREVIATIONS

ADA	Adenosine deaminase
AMP	Adenosine 5'-monophosphate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
3'-AMP	Adenosine 3'-monophosphate
AOPCP	$\alpha\beta$ -methylene adenosine diphosphate
BBS	Borate-buffered saline
BBA	3% (wt/vol) bovine serum albumin in BBS
B-lymphocyte	"Bursa-equivalent" dependent lymphocyte
CLL	Chronic lymphatic leukemia
Con A	Concanavalin A
c.p.m.	Counts per minute
CV-H	"Common variable" hypogammaglobulinaemia
deoxy-ATP	Deoxyadenosine triphosphate
deoxy-GMP	Deoxyguanosine monophosphate
deoxy-TMP	Deoxythymidine monophosphate
E^+ cells	Cells which rosette with sheep erythrocytes
En^+ cells	Cells which rosette with neuraminidase-treated sheep erythrocytes
EDTA	Ethylene diamine tetraacetic acid
Fc	"Crystallisable fragment" of immunoglobulin molecule
FCS	Foetal calf serum
IMP	Inosine monophosphate
Km	Michaelis constant
Bm, Tm	Mitomycin C-treated B or T cells
n.d.	Not determined

5'-N	5'-Nucleotidase
NAD	Nicotinamide adenine dinucleotide
p	Statistical probability
PBS	Phosphate-buffered saline
PHA	Phytohaemagglutinin
PNP	Purine nucleoside phosphorylase
POPOP	p-Bis 2-(5-phenyloxazolyl)-benzene
PPO	2,5 Diphenyloxazole
PWM	Pokeweed mitogen
R.P.M.I.(1640)	Rosewell Park Memorial Institute medium
S	Initial concentration of substrate
SCID	Severe combined immunodeficiency
SEM	Standard error of the mean
s.d.	Standard deviation
sIg^+ cells	Cells with surface immunoglobulin
TBS	Tris-buffered saline
T-lymphocyte	Thymus-dependent lymphocyte
Tris	Tris (hydroxymethyl) methylamine
Vi	Initial velocity of enzyme reaction
Vmax	Maximum velocity of enzyme reaction
X-H	X-linked hypogammaglobulinaemia

INTRODUCTION

5'-Nucleotidase is widely employed as a marker enzyme for the plasma membrane, and little is known of its function *in vivo*. Clinical investigations with the mononuclear cells (lymphocytes and monocytes) of the peripheral blood from certain patients have revealed differences in the level of this enzyme compared with the cells from normal healthy controls. The original investigations with patients with chronic lymphatic leukemia showed that the majority of these patients have extremely low levels of this enzyme (typically $\leqslant$ 10% of the normal values). Later, many patients with adult onset hypogammaglobulinaemia were also found to have low levels of this enzyme; although the deficiency was generally less severe than that observed with chronic lymphatic leukemia. At the time of this study, there was a great deal of uncertainty about the relative 5'-N activities of the constituent subpopulations of the 'circulating mononuclear cells' under investigation. This was obviously a hinderance to any attempt to determine the cause and significance of the low 5'-nucleotidase activity, since the relative proportions of the mononuclear cell subpopulations are known to differ in these patients.

This thesis describes an investigation of the cause and significance of the subnormal 5'-nucleotidase activity found in many patients with hypogammaglobulinaemia. In view of the clinical nature of much of this thesis, the review of the literature on 5'-nucleotidase (chapter 1) includes a brief review of the clinical data relevant to the patients investigated. The experimental section has been presented in three parts (chapters 2, 3 and 4), each with its own technical discussion of the results. A general discussion comprises the final chapter.

CHAPTER 1

A REVIEW OF THE LITERATURE

1.1. 5'-NUCLEOTIDASE (E.C.3.1.3.5.)

1.1.1. HISTORICAL INTRODUCTION

In 1934, Reis investigated the possibility that the liberation of phosphate from nucleotides in cardiac and skeletal muscle may be due to the existence of a group of specific nucleotidases rather than non-specific phosphatases. Reis prepared crude tissue suspensions of various organs from several species and determined the relative rates of phosphate release from adenosine 5'-monophosphate (AMP), adenosine 3'-monophosphate (3'-AMP) and β-glycerophosphate at pH 7.0. Bone and kidney suspensions were found to liberate phosphate at approximately the same rate from all three substrates. However, brain, cardiac muscle, liver and retina suspensions liberated phosphate from AMP more readily than from 3'-AMP and β-glycerophosphate. These results led Reis to postulate the existence of "a phosphatase which is specific for the 5'-monophosphate esters of purine ribosides".

1.1.2. DISTRIBUTION OF 5'-NUCLEOTIDASE

5'-Nucleotidase (5'-N) is a widely distributed enzyme. Shortly after Reis discovered the enzyme in animal tissue extracts, Gulland & Jackson (1938) showed its presence in the venom of many species of snake. In 1954 Wang reported the presence of 5'-nucleotidase in a soil bacterium, and later Kohn & Reis (1963) detected the enzyme in the extracts of many bacteria from human sources. A 5'-N has also been isolated and characterized from the yeast, Saccharomyces oviformis (Takei, Totsu & Nakanishi, 1969). Plant 5'-nucleotidases have been found in extracts of potato (Klein, 1957 a, b) and of leaves (Shuster & Kaplan, 1953).

Although the early work of Reis was conducted with simple tissue extracts, which rendered much of his data approximate, it appears that the

5'-N activity per gram wet weight varies considerably for some tissues from one species to another (Reis, 1940). For example, when inosine 5'-monophosphate was used as substrate at pH 7.6, extracts of rat and horse hearts showed substantially more 5'-N activity per gram wet weight than extracts of hearts from dog, calf, rabbit and pigeon.

Of the components of human peripheral blood, both the serum (Dixon & Purdom, 1954) and lymphocytes (Lopes, Nachbar, Zucker-Franklin & Siber, 1973) have a readily detectable 5'-N activity. Polymorphonuclear leukocytes (Shirley, Wang, De Chatelet & Waite, 1976) and erythrocytes lack the enzyme, although erythrocytes have an enzyme which specifically hydrolyses pyrimidine 5'-nucleotides (Paglia & Valentine, 1975). In contrast, polymorphonuclear leukocytes obtained from guinea pig peritoneal exudates were shown to have nearly ten times more 5'-N activity per cell than the lymphocytes from the same source (De Pierre & Karnovsky, 1974 b). It is not clear whether the difference between human and guinea pig polymorphonuclear leukocytes reflects a species difference or the state of "activation" of the polymorphonuclear leukocyte.

1.1.3. LOCATION OF 5'-NUCLEOTIDASE IN MAMMALIAN CELLS

Early attempts to determine the intracellular location of 5'N in rat liver (Novikoff, Podber, Ryan & Noe, 1953; De Lamirande, Allard & Cantero, 1958) were limited in success owing to the procedures employed to isolate the subcellular organelles. The ultracentrifugal procedures employed by these authors were designed to obtain maximal recovery of the enzyme rather than a high degree of purity of the subcellular organelles. Consequently, cross-contamination of the cellular fractions gave rise to misleading results.

Song & Bodansky (1967) employed isolation procedures which, at the expense of poor recoveries, resulted in subcellular fractions with very little cross contamination. Purification of the nuclear fraction was monitored by the determination of DNA-phosphorus and total protein. They recovered 5% of the protein and 30% of the DNA-phosphorus in their nuclear fraction. Electron microscopic examination revealed the absence of mitochondria, lysosomes, endoplasmic reticulum and membrane vesicles. Recovery of the nuclear marker enzyme, NAD^{+} pyrophosphorylase, was 20%, with a 16-fold increase in specific activity over the starting homogenate. Recovery of the microsomal marker, glucose-6-phosphatase, was less than 0.2%, with a ten-fold decrease in specific activity. The removal of 5'-N from the nuclear fraction paralleled that of glucose-6-phosphatase, indicating that there was little or no 5'-N in the nuclei. A second fraction containing both mitochondria and lysosomes was prepared. About 40% of the mitochondrial marker, glutamate dehydrogenase, and 35% of the lysosomal marker, acid phosphatase, were recovered in this fraction compared with less than 0.6% of the glucose-6-phosphatase and less than 0.4% of the 5'-N. These findings suggested that 5'-N was not present in mitochondria or lysosomes. Finally, a microsomal fraction was prepared in which the recoveries of 5'-N and glucose-6-phosphatase were 23% and 27% respectively. Although this microsomal fraction was slightly contaminated with lysosomes and mitochondria (7.5% and 2% recovery of their respective marker enzymes), the high and parallel yields of glucose-6-phosphatase and 5'-N suggested that both enzymes were exclusively present in the microsomes.

The term "microsome" is used to describe the ultracentrifugal preparation which consists of plasma membrane and endoplasmic reticulum

fragments, with possible contamination from other cell components. Sucrose density gradient centrifugation of the rat liver microsomal fraction revealed that the 5'-N activity resided in the membrane fraction of the microsomes rather than the ribosomes (Song & Bodansky, 1967). Further purification of the plasma membrane by Song & Bodansky (1967) indicated that most of the microsomal 5'-N was derived from the plasma membrane rather than the endoplasmic reticulum.

In 1969, Fritzson partially purified a cytoplasmic 5'-N from rat liver. As will be discussed later, this enzyme had different properties to the membrane-bound enzyme. The general properties of Fritzson's cytoplasmic enzyme suggested that it was identical to the 5'-N isolated from liver acetone powder preparations by Itoh, Mitsui & Tsushima (1968).

As was pointed out by De Pierre & Karnovsky (1973), the use of solid organs, such as liver, for subcellular fractionation studies poses problems. Solid organs are generally composed of more than one cell type and are vascular, with nerve processes innervating the blood vessels. With the low yields of plasma membrane generally obtained, there is a danger that the preparations may be enriched with material from a minor cell type. This problem can be avoided by studying single cell suspensions. Subcellular fractionation studies by Bosman, Hagopian & Eylar (1968), using HeLa cells, confirmed the findings of Song & Bodansky (1967), showing that 5'-N was located in the plasma membrane. Cytochemical studies have also been used to demonstrate the location of 5'-N in the plasma membrane of rat lymphocytes (Misra, Gill & Estes, 1974). However, the use of cytochemical staining alone to demonstrate the location of 5'-N should be viewed with caution (De Pierre & Karnovsky, 1973).

Uusitalo & Karnovsky (1977a) have used both cytochemical staining and biochemical analysis of disrupted cells to demonstrate that 5'-N is almost exclusively located in the plasma membrane of mouse lymphocytes. Despite the presence of a 5'-N in liver that is not membrane-bound, it appears that most of the enzyme in mammalian cells is found in the plasma membrane. Indeed, the enzyme is now commonly employed as a plasma-membrane marker (De Pierre & Karnovsky, 1973).

De Pierre & Karnovsky (1974a) have shown that the active sites of membrane-bound 5'-N of the guinea pig polymorphonuclear leukocyte face the external medium rather than the cytoplasm. This conclusion was derived from three types of experiments. First, the 5'-N activity of intact cells was not increased by disruption of the cell membrane which acts as a permeability barrier to AMP. Second, the concentration of inorganic phosphate produced from AMP by intact cells was shown to be 100 times greater in the extracellular medium than in the cells. The possibility that the substrate was hydrolysed intracellularly, and the products transported out, was dismissed by preloading the cells with (^{33}P) phosphate and using (^{32}P) AMP as substrate. Third, the cells were treated with the diazonium salt of sulphanilic acid which cannot penetrate intact cells. This treatment rapidly inhibited intact cell 5'-N activity. These experiments provided strong evidence that 5'-N was an "ecto-enzyme".

1.1.4. PURIFICATION AND PROPERTIES OF MAMMALIAN 5'-NUCLEOTIDASE

Purification

In 1951, Heppel & Hilmoe obtained a 50-fold purification of 5'-N from bull seminal plasma. The seminal plasma was first treated with protamine sulphate, a polycation which, through its charged groups,

precipitates polyanions such as nucleic acid proteins. The supernatant from the protamine sulphate step was then subjected to two ammonium sulphate precipitation steps, followed by two fractionations with ethanol. At this stage, Heppel & Hilmoe utilized the stability of the 5'-N to remove contaminating non-specific phosphatase activity: the preparation was heated to 60°C for twenty minutes and, after rapid cooling, adsorbed on aluminium hydroxide gel. The final preparation was free of non-specific phosphatase activity and contained 4.1% of the original 5'-N activity.

A similar procedure was employed by Song and Bodansky (1966) to obtain a preparation of 5'-N from human liver that was free of non-specific phosphatase activity. The location of the 5'-N in the plasma-membrane of liver cells necessitated a solubilization step using 3% sodium deoxycholate. Otherwise, the procedure was essentially that of Heppel & Hilmoe (1951). A ten-fold increase in the specific activity was obtained, with a yield of 15%.

Two major modifications to the above procedure enabled Widnell & Unkeless (1968) to obtain a 5'-N fraction which contained only one major lipoprotein. Rather than solubilizing the enzyme from the rat liver homogenate, they first isolated plasma-membranes: this alone can produce a 12-fold increase in specific activity (Song and Bodansky, 1967) although the yield may be poor. The solubilized plasma-membranes were fractionated with ammonium sulphate, heated to 50°C for 5 minutes, and refractionated with ammonium sulphate. Agarose column chromatography was used for the final separation. A more elaborate scheme of column chromatography by Nakamura (1976) resulted in purification of 5'-N to a single protein which again contained phospholipid. After ammonium sulphate fractionation and

heat-treatment of solubilized rat liver plasma-membranes, Nakamura further purified 5'-N by DEAE-cellulose chromatography and affinity column chromatography with 5'-AMP-Sepharose.

5'-N was isolated as a single, lipid-free, protein from mouse liver plasma-membranes by Evans & Gurd in 1973. These authors used a detergent buffer, N-dodecylsarcosinate-Tris, to solubilize the membrane proteins which were then fractionated by a rate-zonal centrifugation in a sucrose-detergent gradient. The 5'-N activity peak, which was associated with 5% of the protein applied to the gradient, was further purified by gel filtration on Sepharose and Sephadex columns. The purified enzyme was found to be a glycoprotein of molecular weight 140,000 - 150,000, which appeared to be composed of two glycoproteins of identical molecular weight (70,000 - 75,000). The molecular weight determined for the enzyme corresponded closely to the value of 140,000 obtained in the absence of detergent with sheep brain 5'-N (Ipata, 1968), and has since been confirmed with the plasma membrane enzyme from other sources (Nakamura, 1976; Dornand, Bonnafous & Mani, 1978).

Properties of mammalian 5'-nucleotidase

Evans and Gurd (1973) proposed that the large amounts of sphingomyelin which Widnell and Unkeless (1968) obtained with their purified enzyme were mainly associated with detergent micelles, and were not the result of specific lipid-protein interactions that may occur in the intact cell membrane. Addition of pure phospholipids to the 5'-N isolated by Evans & Gurd did not increase the specific activity of the enzyme. This does not, however, rule out the possibility of specific lipid-protein interactions *in situ*, since the added phospholipids may have been unable to replace or exchange with the detergent.

The substrate specificity of the enzyme purified from mouse liver membranes by Evans & Gurd is shown in table 1. These results are similar to those obtained by other authors for the plasma membrane enzyme (Song & Bodansky, 1966; Widnell & Unkeless, 1968; Nakamura, 1976; Dornand, Bonnafous & Mani, 1978). The cytoplasmic 5'-N of rat liver differs in many respects to the membrane-derived enzyme. Both inosine 5'-monophosphate (IMP) and deoxy-guanosine 5'-monophosphate (deoxy-GMP) are better substrates for the cytoplasmic enzyme than is AMP (table 2). The Michaelis constant (Km) of the cytoplasmic enzyme for AMP substrate is about 6.8 mM (Fritzson, 1969), which is more than 100-fold greater than the values determined for the membrane enzyme (Song & Bodansky, 1967; Widnell & Unkeless, 1968; Evans & Gurd, 1973; Nakamura, 1976). A 5'-N has also been partially purified from sheep brain (Ipata, 1968) and rat heart (Sullivan & Alpers, 1971) using procedures which suggest that the enzyme was of cytoplasmic origin. However, the substrate specificity and general properties corresponded to those of the membrane-derived enzyme. It is therefore tempting to speculate that the 5'-N of the liver cytoplasm differs from the enzyme found in the membranes and cytoplasm of cells from other tissues, and may perform a function that is unique to liver cells.

There is some confusion in the literature regarding the pH optima of 5'-N. Generally, it appears that the membrane enzyme has two optima; one between pH 7.0 - 7.5, and a second between pH 9.0 - 10.0 (Bodansky & Schwartz, 1963; Song & Bodansky, 1966, 1967; Evans & Gurd, 1973). The latter pH optimum is dependent upon the presence of Mg^{++} ions in the assay. Apart from the effect of Mg^{++} ions, the choice of buffer has also been shown to be important (Bodansky & Schwartz, 1963; Burger & Lowenstein, 1970). In particular, glycine inhibits 5'-N below pH 7.5 and activates

TABLE 1

SUBSTRATE SPECIFICITY OF 5'-NUCLEOTIDASE PURIFIED FROM MOUSE LIVER PLASMA MEMBRANES

Substrate	Relative Activity	
	pH 7.6	pH 10.0
Adenosine 5'-monophosphate	100	100
Uridine 5'-monophosphate	105	117
Cytidine 5'-monophosphate	70	60
Guanosine 5'-monophosphate	88	49
Adenosine 3'-monophosphate	0	n.d.
Adenosine 2'-monophosphate	0	n.d.
Thymidine p-nitrophenyl phosphate	0	13
p-Nitrophenyl phosphate	0	0
Adenosine triphosphate	0	n.d.

Taken from Evans & Gurd (1973). Results are expressed as a percentage of the specific activity obtained with adenosine 5'-mono phosphate as substrate. (n.d. = not determined).

TABLE 2.

SUBSTRATE SPECIFICITY OF THE CYTOPLASMIC 5'-NUCLEOTIDASE PARTIALLY PURIFIED FROM RAT LIVER

Substrate	Relative activity at pH 6.5	
	Ref. (a)	Ref. (b)
Inosine 5'-monophosphate	100	100
deoxy-GMP	98	90
Uridine 5'-monophosphate	50	38
Adenosine 5'-monophosphate	36	17
deoxy-TMP	16	n.d.

Data taken from: ref. (a), Fritzson (1969); ref. (b), Itoh, Mitsui, & Tsushima (1968). Results are expressed as a percentage of the specific activity obtained with inosine 5'-monophosphate as substrate.

above this pH. The practice of some authors of using different buffer systems for a series of points on the same pH profile is therefore open to criticism. It is now apparent that this was the reason for Heppel & Hilmoe (1951) observing a single pH optimum at pH 8.0 with bull semen plasma 5'-N: Bodansky & Schwartz (1963) and Levin & Bodansky (1966) have since shown that the enzyme from this source has two pH optima.

Not all the discrepancies in the literature may be explained by the assay conditions employed. The cytoplasmic 5'-N of liver has a single optimum at around pH 6.5 (Itoh, Mitsui and Tsushima, 1968; Fritzson, 1969). A partially purified 5'-N from pig gut has been observed to have three pH optima; at 5.5, 7.0 and 8.5 (Burger & Lowenstein, 1970). Although the isolation procedure employed by Burger & Lowenstein was such that the final enzyme may well have been a mixture of both cytoplasmic and membrane-derived 5'-N, this does not seem the likely cause of the optimum at pH 5.5. Fox & Marchant (1976) observed a single optimum at pH 9.8 with the membrane-bound 5'-N of human placenta in the absence of Mg^{++} ions, and a broad plateau of activity between pH 7.4 - 9.8 in the presence of Mg^{++} ions. Dornand, Bonnafous & Mani (1978) observed a single optimum at pH 7.5 with 5'-N purified from pig lymphocyte plasma membrane, regardless of whether Mg^{++} ions were present during the assay. It is interesting to note that Dornand et al found the purified enzyme to have a different pH profile to that of the enzyme present in intact membranes.

5'-N appears to be a Mg^{++}-dependent enzyme. The cytoplasmic enzyme of rat liver is completely inactive in the absence of divalent cations, of which Mg^{++} is the most effective activator but Ca^{++} has no effect (Itoh et al, 1968). The enzyme from other sources appears to have a tightly bound divalent ion; no additional Mg^{++} is necessary for activity at

pH 7.4, but EDTA reversibly inhibits the enzyme (Emmelot & Bos, 1966; Sullivan & Alpers, 1971; Dornand, Bonnafous & Mani, 1978). Mg^{++} and Mn^{++} ions proved to be the most effective at reversing the inhibition of EDTA. The second, alkaline, pH optimum reported for 5'-N is dependent upon the presence of Mg^{++} even in the absence of EDTA (Song & Bodansky, 1966, 1967; Evans & Gurd, 1973). Mg^{++} ions have also been shown to reverse the inhibition of 5'-N by ADP and ATP (Burger & Lowenstein, 1970; Sullivan & Alpers, 1971).

Nucleoside di- and tri- phosphates are competetive inhibitors of 5'-N activity; the diphosphates being the more powerful inhibitors (Burger & Lowenstein, 1970; Sullivan & Alpers, 1971; Nakamura, 1976; Rodan, Bourret & Cutler, 1977; Dornand, Bonnafous & Mani, 1978). The phosphonate analogue of ADP, $\alpha\beta$-methylene adenosine diphosphate (AOPCP), is an even more potent inhibitor of the enzyme (Burger & Lowenstein, 1970, 1975). Ipata (1968) reported that a 5'-N partially purified from sheep brain was allosterically inhibited by ATP. However, no other reports of sigmoidal inhibition curves are known; indeed, Burger & Lowenstein (1975) were unable to reproduce the work of Ipata.

1.1.5. THE ASSAY OF 5'-NUCLEOTIDASE ACTIVITY

Until recently, 5'-N activity was commonly measured by the calorimetric determination of inorganic phosphate (Pi) released from the nucleotide substrate. This procedure does not allow the inclusion of competetive inhibitors of non-specific phosphatase activity, such as β-glycerophosphate or 3'-AMP, in the assay. Consequently, the contribution of non-specific phosphatases to the breakdown of the 5'-monophosphates must be determined in a separate assay (eg: Reis, 1934; Dixon & Purdom, 1954).

Ipata (1967, 1968) employed a quicker procedure which involved coupling the 5'-N to the deamination of adenosine, by the presence of excess adenosine deaminase, and following the reaction spectrophotometrically at 265 nm. In this system, β-glycerophosphate may be added to inhibit non-specific alkaline phosphatase activity (Belfield & Goldberg, 1968).

The above methods are laborious and prompted the development of radiometric procedures which were quicker and more sensitive. Thin-layer chromatography (Shenoy & Clifford, 1975), high-voltage electrophoresis (Fox & Marchant, 1976; Webster et al, 1978) and ion-exchange chromotography (Chatterjee, Bhattacharya & Barlow, 1979) have been employed to separate the products of the 5'-N reaction from the substrate. Adsorption of unreacted ^{32}P-labelled AMP onto Norit A (Quagliata, Faig, Conklyn & Silber, 1974) or onto charcoal (De Pierre & Karnovsky, 1974a) also allows a quick and sensitive assay, but suffers a serious limitation in that the ^{32}P has a short half-life of only 14 days. Methods which employ ^{14}C or ^{3}H labelled substrate do not have this drawback. The procedure used in this thesis was based on that described by Avruch & Wallach (1971) in which $ZnSO_4$ - $Ba(OH)_2$ was used to quantitatively precipitate AMP, leaving adenosine in solution.

5'-N may also be assayed by using histochemical methods based on that developed for the demonstration of phosphatase activity in tissue sections (Gomori, 1939). This system locates the site of enzyme activity by precipitation of the Pi reaction product with metal ions. Usually, lead ions are used and the precipitate is converted to brown lead sulphide for visualization by light microscopy, although the system can be adapted for electronmicroscopy. Histochemical assays for 5'-N have been employed in the investigation of the enzyme on human lymphocytes (Müller-Hermelink, 1974; Silber, Conklyn, Grusky & Zucker-Franklin, 1975).

While the histochemical assays have proved useful in the studies of lymphocyte 5'-N, the limitations and drawbacks should be appreciated. The formation of a lead precipitate at the site of reaction is dependent upon the rate of production of Pi, and the efficiency of the trapping of the Pi by the lead ions. De Jong, Hak, Van Duijn & Daems (1979) used a model system to show that the trapping efficiency was reduced by a number of factors commonly present in the media employed in many assay systems. By taking into account the rate of enzyme reaction in various incubation media, together with the trapping efficiency, De Jong et al were able to modify the conditions to obtain more than double the percentage of cells staining positively for acid phosphatase than when using the media employed by other workers. The same principles are applicable to the histochemical determination of 5'-N. It is therefore apparent that the 'threshold' for the positive demonstration of enzyme activity in a cell is dependent on the composition of the incubation medium. This should be taken into consideration when attempting to define subpopulations that are 'positive' or 'negative' for 5'-N. When using the histochemical assay for determination of the cellular location of 5'-N (eg, Misra, Gill & Estes, 1974) one should also consider the possibility that the lead precipitate may not be located at the site of the enzyme.

1.1.6. MECHANISM OF 5'-NUCLEOTIDASE ACTIVITY

Site of cleavage of substrate

By studying the hydrolysis of AMP by bull semen plasma 5'-N in the presence of H_2O^{18}, Koshland & Springhorn (1956) showed that the phosphate was liberated by cleavage of the P-O rather than the C-O bond:

$$\text{Ad-O } PO_3 + H_2O^{18} \longrightarrow \text{Ad-OH} + HO^{18}PO_3$$

Mechanism of 5'-nucleotidase activity

Little is known about the mechanism of 5'-N activity although Levin & Bodansky (1966) postulated a model that was consistent with their findings regarding the effect of Mg^{++} ions and pH. They proposed that 5'-N has four sites relevant to its catalytic activity (see Fig. 1). The binding sites for the nucleotide and H_2O are denoted by X and W respectively. Site Y is a cationic binding site; at low pH values it is designated Y-H^+, and at alkaline pH it is converted to Y which allows combination with Mg^{++}. The fourth relevant to proposed mechanism is designated Z, and the active form of this site is Z-H^+, which binds the phosphate moiety of the nucleotide substrate.

They proposed that Z-H^+ binds with the phosphate moiety of the substrate molecule; which induces a positive charge on the phosphorus atom and facilitates attack by the hydroxyl ion present at the water-binding site, W. As the pH is raised, Z-H^+ is inactivated. In the presence of Mg^{++}, raising the pH results in replacement of H^+ with Mg^{++} at the electronegative site, Y. The Y-Mg^{++} complex then binds with the phosphate. This makes the phosphate moiety more electrophilic and thus more susceptible to interaction with the hydroxyl ion at site W. This model offers a plausible explanation for the double pH optimum of 5'-N.

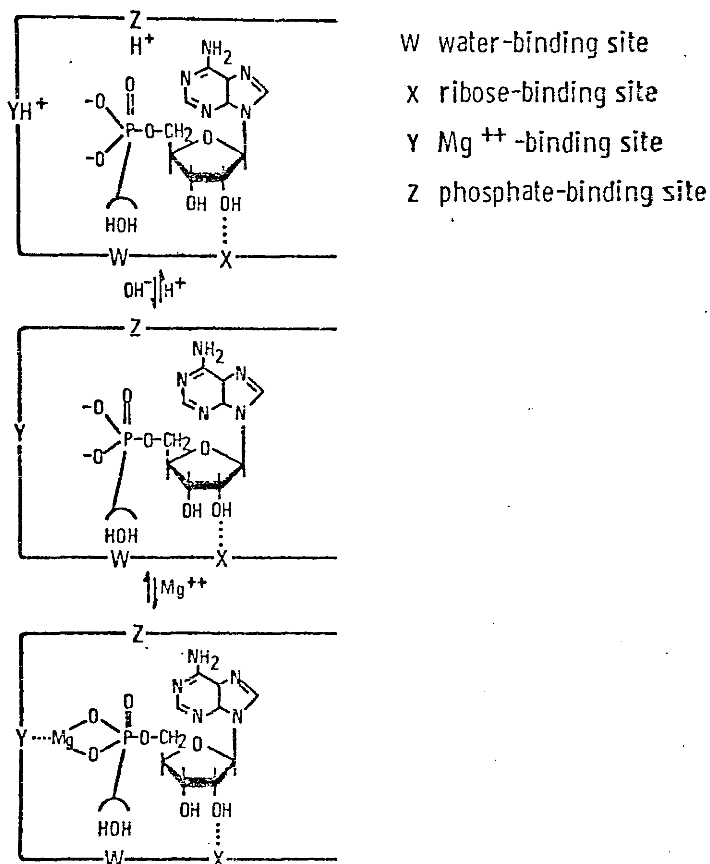
1.1.7 THE PHYSIOLOGICAL FUNCTION OF 5'-NUCLEOTIDASE

The role of 5'-N is unclear. At least four important enzymes in the glycolytic pathway and citric acid cycle are influenced by the concentration of AMP (Atkinson, 1966). The 'adenylate control hypothesis' thus proposes that glycogen phosphorylase, phosphofructokinase, fructose-1,6-diphosphatase, and isocitrate dehydrogenase may be under

Figure 1

MECHANISM OF 5'-NUCLEOTIDASE ACTIVITY

Proposed model for the effect of Mg^{++} on the hydrolysis of AMP by 5'-Nucleotidase (from Levin & Bodansky, 1966)



the influence of 5'-N activity. More recently, Arch & Newsholme (1978) reviewed the evidence for a hormonal role of adenosine, and the potentially important role of 5'-N in regulating the concentration of adenosine. These authors considered that the ability of Mg^{++} to relieve the inhibition of 5'-N by ADP and ATP was important for the regulation of the enzyme in situ.

The regulatory function of 5'-N mentioned above may well be of importance for the cytoplasmic enzyme but the role of the ecto-enzyme remains enigmatic. It has been proposed that the function of human lymphocyte ecto-5'-N is the salvage of purines from AMP (Fleit, Conklyn, Stebbins & Silber, 1975). The cell membrane is impermeable to nucleotides but permeable to nucleosides; thus the enzyme may facilitate the uptake of purines from nucleotides in the extracellular medium. However, as pointed out by the authors themselves, it remains uncertain whether an absence of ecto-5'-N presents a physiological disadvantage to the cell in vivo, since phosphatases (and 5'-N) in the surrounding environment would cleave the nucleotides and make the nucleoside available to the cell.

5'-N has been reported to reverse the inhibitory action of actin on deoxyribonuclease by interacting with actin (Mannherz & Rohr, 1978). In this respect, it is interesting that actin has been reported on the surface of lymphocytes, and mainly on B-lymphocytes (Owen et al, 1978). However, the significance of these observations in relation to the function of 5'-N is not known.

Cohn & Parks (1967) found that high concentrations of AMP, ADP, ATP and to a lesser extent, adenosine, induced the formation of large numbers of vesicles in mouse macrophages, while other nucleotides and related nucleosides had little or no effect. This stimulation of pinocytosis was later observed to be paralleled by a dramatic increase in ecto-5'-N activity (Lazdins & Karnovsky, 1978). If this increase in 5'-N activity were to

result in an increase in the intracellular nucleoside and nucleotide pools, there may well be an increase in the cell metabolism. Cohn & Parks (1967) raised the question of whether such an increase in cell metabolism might cause the cell to respond more vigorously to an external stimulus (the response being pinocytosis).

1.1.8. HUMAN LYMPHOCYTE 5'-NUCLEOTIDASE

Normal human lymphocytes have a readily detectable 5'-N activity (Lopes, Nachbar, Zucker-Franklin & Silber, 1973). The enzyme is located in the plasma membrane and the observation that Concanavalin A, which binds to cell surface glycoproteins, inhibits up to 90% of the 5'-N activity of lymphocytes (Webster et al, 1978) is compatible with the cell-surface location of the enzyme. Although Muller-Hermelink (1974) showed that B-cell regions of lymphatic tissue sections stained positively for 5'-N activity, while the T-cell regions were negative, other workers have failed to demonstrate a significant difference between the level of 5'-N activity on B- and T- cells (Quagliata et al, 1974; Kramers et al, 1976; Edwards, Magilavy, Cassidy & Fox, 1978).

That many, but not all, patients wich chronic lymphatic leukemia (CLL) have markedly reduced levels of 5'-N on their circulating lymphocytes is now well documented (Lopes, Zucker-Franklin & Silber, 1973; Marique & Hildebrand, 1973; Quagliata et al, 1974; Kramers et al, 1976; La Mantia, Conklyn, Quagliata & Silber, 1977; Kanter, Freiberger, Rai & Sawitsky, 1979). A minority of CLL patients have supranormal levels of 5'-N activity (Quagliata et al, 1974; La Mantia et al, 1977; Kanter et al, 1979). Cord blood lymphocytes were reported to have less 5'-N activity than the blood lymphocytes from healthy adults (Kramers, Catovsky, Cherchi & Galton, 1977).

The circulating mononuclear cells of patients in the early stages of infectious mononucleosis also have a low level of 5'-N activity, but otherwise the 5'-N activity has been shown to be unaffected in a number of cardiopulmonary, metabolic, neoplastic, dermatological, and infectious disorders (Quagliata et al, 1974).

Soon after the discovery that certain immunodeficiency diseases were associated with defects of purine metabolism (see 1.2.6.), Johnson et al (1977) reported that many patients with late onset 'common-variable' hypogammaglobulinaemia(CV-H) have low levels of 5'-N on their circulating mononuclear cells. This led to the report that patients with X-linked hypogammaglobulinaemia (X-H) had 5'-N activities at the lower limit of the normal range, and that three patients with selective IgA-deficiency had barely detectable 5'-N activity (Webster et al 1978). A number of other enzymes involved in purine metabolism were found to have normal activity in patients with hypogammaglobulinaemia (Webster et al, 1978). A group of American workers have obtained different results with their patients. They reported that their group of X-H patients had a lower 5'-N activity than the patients studied by Webster et al, and that their CV-H patients had an apparently normal range of 5'-N activity, as did their patients with IgA-deficiency (Edwards et al, 1978).

The primary aim of the work presented in this thesis was to investigate the cause and significance of low 5'-N activity in patients with hypogammaglobulinaemia. In order to gain an understanding of why certain lines of thought were pursued, there follows a brief review of the type of patients studied and the association of defects of purine metabolism with certain immunodeficiency diseases.

1.2. PRIMARY IMMUNODEFICIENCY

1.2.1. INTRODUCTION

Immunological responses are classically divided into 'humoral' and 'cell-mediated' type responses: both of which are lymphocyte-dependent. B-lymphocytes, with the help of T-cells, are responsible for the synthesis and secretion of free antibody into the blood and other body fluids (humoral antibody), while the T-lymphocytes are the mediators of specific cellular immunity.

Historically, X-linked hypogammaglobulinaemia (X-H) was the first type of immunodeficiency to be recognized (Bruton, 1952). This condition represents a pure B-cell defect, with impairment of antibody and immunoglobulin synthesis (i.e. the humoral response). The DiGeorge syndrome (DiGeorge, 1965, 1968), in which the cell-mediated response is undetectable, represents a pure T-cell defect. Although a humoral response can be elicted in the DiGeorge syndrome, it is subnormal since the B-cells require the co-operation of T-cells. Severe combined immunodeficiency (SCID) arises from the failure of both B- and T- cells to develop normally, with a consequent loss of both humoral and cell-mediated immunity. The three aforementioned types of immunodeficiency are the most clear-cut, and also rare, examples recognized. The majority of immunodeficient patients defy such clear-cut classification and are grouped under the heading of 'common-variable' immunodeficiency.

1.2.2. X-LINKED HYPOGAMMAGLOBULINAEMIA

Patients with X-H have a defect of humoral immunity and are subject to recurrent bacterial infections. They handle most viral infections normally, although they are prone to Echovirus infection which produces a

mild self-limiting illness in normal individuals but is usually fatal in patients with X-H (Wilfert, 1977). Respiratory symptoms nearly always present in the first two years of life, and usually after the first two months. Antibodies passively transferred in utero probably provide adequate protection in the first few months of life.

The disease is characterised by low levels of all classes of immunoglobulin in the serum. Frøland & Natvig (1972) first observed that these patients lacked circulating B-cells, but it is now clear that some patients may have small numbers of these cells (Geha, Rosen & Merler, 1973; Schwaber, Lazarus & Rosen, 1978). Although it is possible that these patients have B-cells that differ from normal in that they lack surface immunoglobulin, a number of studies suggest that this is unlikely to be the case. Hoffman et al (1977) showed that X-H patients lack lymphocytes with another B-cell marker, Ia-type antigen, on their surface. Geha, Rosen & Merler (1973) demonstrated a lack of cells with the density of B-cells in the blood of these patients. Finally, Wiig (1975) showed that cells with the electrophoretic mobility of B-cells were missing.

The bone marrow of X-H patients contains normal numbers of pre-B cells, although their rate of division is reduced (Vogler et al, 1976; Pearl et al, 1978). The presence of pre-B cells and the low, but definite, level of serum immunoglobulin in patients with X-H suggest that the disease is not due to a severe defect in the structural gene for immunoglobulin. It is possible that there is a maturation arrest at the pre-B stage.

These patients appear to have normal T-cells and normal cell-mediated immunity. The blastogenic response to Phytohaemagglutinin (PHA) and antigens is normal (Gotoff, 1968). In addition, the total numbers of T-cells are

normal, as is the proportion with receptors for IgM and IgG (Moretta et al, 1977; Gupta & Good, 1977).

Inheritance is of the classical sex-linked type in which the 'carrier' female has one normal X-chromosome and one containing the defective allele. Thus, the affected males have the defective gene on their single X-chromosome. Of the patients' offspring, the sons will be normal but the daughters become 'carriers'. Owing to the small sizes of families, diagnosis of the disease is often uncertain; but it is highly probable when there are affected brothers, and almost certain when there is an affected maternal uncle.

Treatment of these patients involves regular injections of gamma-globulin, which helps to prevent serious bacterial infections. Provided that these patients receive regular medical care, with early treatment of any infections, their prognosis is reasonable.

1.2.3. SEVERE COMBINED IMMUNODEFICIENCY

Although no patients with this syndrome were studied in this thesis, they are of interest because of the association with defects of purine metabolism (see 1.2.6.). They have a severe defect of both cellular and humoral immunity, and suffer recurrent bacterial, viral, and fungal infections. SCID characteristically presents in the first three months of life and is fatal if untreated. The syndrome may show autosomal recessive or X-linked inheritance, or may occur sporadically.

Lymphopenia is a common feature of this disease, but the number of B-cells is often normal or raised (Siegal & Good, 1977). The classes of immunoglobulin on the B-cells of these patients may be unusual (Preud'homme, Clauvel & Seligmann, 1975) and antibody production is invariably lacking.

Until recently, the cause of SCID was believed to be a stem cell defect in the bone marrow that prevented the natural differentiation into B- and T- cells. However, since most patients are observed to have some B-cells in their blood, this is unlikely to be a common cause of the disease. The discovery that a deficiency of the enzyme, adenosine deaminase (ADA), is the cause of the disease in a number of SCID patients (see 1.2.6.) has strengthened the belief that a biochemical abnormality may be interfering with the maturation of both B- and T- cells.

1.2.4. SELECTIVE IgA-DEFICIENCY

Severe IgA-deficiency in the serum is puzzling in that it has a relatively high incidence of about 1/700 among the healthy population of many European communities (Koistinen, 1975; Holt, Tandy & Anstee, 1977). The role of IgA is uncertain. Since IgA is the major immunoglobulin on mucosal surfaces, measurement of IgA levels in mucosal secretions may be more relevant than serum IgA levels. However, with the rare exceptions, severe deficiency of serum IgA is paralleled by a deficiency of secretory IgA (Goldberg, Douglas & Fudenberg, 1969; Cassidy, Oldham & Platts-Mills, 1979). Respiratory infections and autoimmune-diseases are found in a number of patients with severe IgA-deficiency (Amman & Hong, 1971). However, because of the high incidence of IgA-deficiency among the healthy population, and the problems arising from the selection of patients in a hospital series, it is difficult to assess the association between IgA-deficiency and disease.

A deficiency of IgA does not appear to be crucial in Europeans, but the rarity of this immunoglobulin deficiency among Africans (Lawton, Royal, Self & Cooper, 1972) suggests that it may be necessary for survival in adverse conditions. It appears that although IgA may provide an important defence against bacterial infection, its absence is usually compensated for by an increased efficiency of one or more of the other components of the immune system. The replacement of IgA by IgM in secretions may be important in preventing infection, although respiratory infections have been observed in an IgA-deficient patient with adequate compensatory IgM (personal communication, A.D.B. Webster).

Patients with severe IgA-deficiency appear to have normal functional T-cells. In particular, the T-cells provide normal 'help' for the production of IgA by normal B-cells (Cassidy, Oldham & Platts-Mills, 1979). In contrast, the B-cells of these patients fail to synthesize IgA even when separated from their own T-cells and provided with normal T-cell help. Synthesis of IgM by the B-cells of these patients is normal, but IgG synthesis is sometimes subnormal.

1.2.5. 'COMMON-VARIABLE' HYPOGAMMAGLOBULINAEMIA

Patients with CV-H are usually grouped into childhood- or adult-onset CV-H, depending on whether the onset of symptoms occurred before or after the patients sixteenth birthday. This avoids the possibility of including any X-H patients in the adult-onset group. However, the two groups of CV-H patients are very similar clinically (Asherson & Webster, 1980). The majority of CV-H patients develop their disease in the third decade. In common with X-H patients, CV-H patients suffer recurrent respiratory infections and handle most viral infections normally. However, they differ

from the X-H patients in that they tend to develop a number of unusual complications such as polyarthritis and autoimmune disease. Most patients with CV-H respond well to weekly injections of gammaglobulins and treatment with antibiotics.

The number of T-cells is normal in CV-H patients, but about a third of the patients show a T-cell defect as indicated by a negative delayed hypersensitivity skin test and a poor *in vitro* response to PHA (Webster & Asherson, 1974). However, these patients do not suffer more infections than those with normal T-cell function; which renders the relevance of these tests to the *in vivo* function of the cells questionable. Suppressor T-cells are present in some patients, although patients with suppressor T-cells normally lack circulating B-lymphocytes (de Gast et al, 1979). The significance of suppressor T-cells in CV-H is therefore unclear. Circulating B-lymphocytes are absent in about a quarter of CV-H patients, and low in a further quarter (Preud'homme, Griscelli & Seligmann, 1973; Horwitz, Webster & Newton, 1977). When present, they consistently show poor immunoglobulin in response to the *in vitro* stimulation with Pokeweed mitogen (PWM), even when provided with normal T-cell help (De la Concha et al, 1977).

With the majority of patients with CV-H there is no convincing evidence for a hereditary basis to the disease. However, Freidman et al (1977) have shown an increased incidence of autoimmune phenomena in relatives of patients with CV-H. It is therefore possible that some cases of CV-H may arise from environmental influences on a predisposing genetic background.

1.2.6. ENZYME DEFECTS IN IMMUNODEFICIENCY DISEASE

1.2.6.1. Introduction

The discovery of two unrelated children with SCID who lacked ADA activity in their erythrocytes (Giblett et al, 1972) provided the first clue that a biochemical defect may be responsible for an immunodeficiency disease. Shortly after this discovery, Giblett et al (1975) reported a deficiency of the enzyme, purine nucleoside phosphorylase (PNP), in a child with a predominant T-cell defect. Both these enzymes are components of the purine interconversion pathway (Fig. 2).

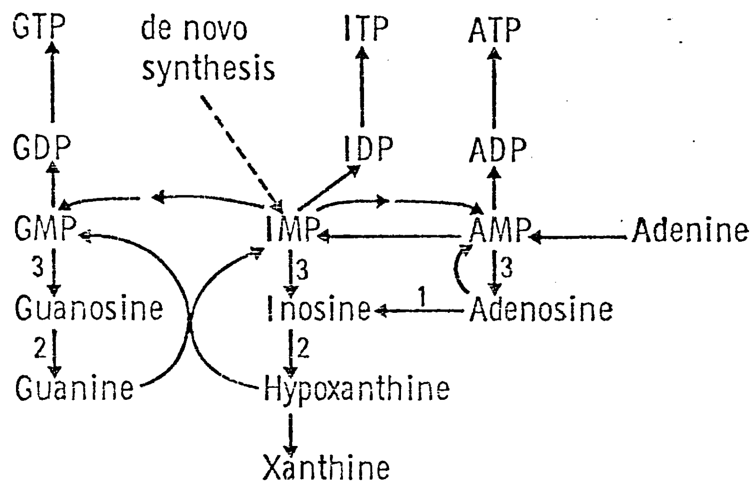
1.2.6.2. Adenosine deaminase deficiency

ADA catalyses the conversion of adenosine or deoxyadenosine to inosine or deoxyinosine respectively, and is present in most tissues of the body. Parkman et al,(1975) presented evidence suggesting that ADA-deficiency is transmitted as an autosomal Mendelian trait. Thus homozygosity of a 'silent' gene for ADA causes a severe deficiency of the enzyme. Clinically, SCID patients lacking ADA resemble those with the enzyme (Meuwissen, Pollara, & Pickering, 1975).

The consequences of ADA-deficiency are unclear. Green & Chan (1973) suggested that cell proliferation was inhibited as a result of an increase in the intracellular concentration of adenosine, which ultimately leads to pyrimidine starvation. Wolberg et al (1975) proposed that ADA-deficiency resulted in elevated levels of cyclic-AMP, which may have a more direct suppressive effect on the immune-function of lymphocytes. A more recent view is that the accummulation of the toxic metabolite, deoxy-ATP, is responsible for the loss of lymphocyte function in ADA-deficient patients.

Figure 2

PURINE INTERCONVERSION PATHWAYS



1. Adenosine deaminase (ADA, EC 3.5.4.4)
2. Nucleoside phosphorylase (PNP, EC 2.4.2.1)
3. 5'-Nucleotidase (5'-N, EC 3.1.3.5)

Deoxy-adenosine has been found in raised concentrations in the urine of these patients (Simmonds, Sahota, Potter & Cameron, 1978), and such concentrations of this nucleoside inhibit the *in vitro* blast-transformation of normal lymphocytes in the presence of an inhibitor of ADA (Simmonds, Panayi & Corrigall, 1978). The absence of ADA in lymphocytes results in the conversion of deoxy-adenosine to deoxy-ATP by a purine kinase (Carson, Kaye & Seegmiller, 1977). High concentrations of deoxy-ATP have now been demonstrated in the lymphocytes of ADA-deficient patients (Cohen, Ullman, Gudas & Martin, 1979) and it has been suggested that the toxicity of this metabolite is due to the fact that it inhibits the enzyme, ribonucleotide reductase, which may be important in controlling DNA synthesis.

Although bone marrow transplants remain the only current cure for SCID, enzyme replacement therapy has proved successful for treating a number of ADA-deficient patients (Polmar, 1979). Transfusion of red cells, which contain ADA and have a half-life of about four weeks, causes an increase in the number of circulating T-cells and a restoration of blast transformation response to PHA. The lymphocyte ADA activity does not increase, and the treatment probably works by reducing the concentration of toxic metabolites in the serum.

1.2.6.3. Purine nucleoside phosphorylase deficiency

PNP catalyses the conversion of inosine or guanosine to their corresponding bases. No corresponding enzyme has been found to convert adenosine to adenine in human tissues. A deficiency of PNP has been reported in seven children from four families (Giblett et al, 1975; Stoop et al, 1977; Biggar et al, 1978 ; Virelizier et al, 1978). All

these patients had defective T-cell function but normal, or raised levels of serum immunoglobulin. This is in contrast to ADA-deficiency which affects both T- and B-cell function. Plasma and urinary concentrations of inosine, deoxy-inosine, guanosine and deoxy-guanosine were found to be raised in PNP-deficient patients (Stoop et al, 1977; Simmonds, Sahota, Potter & Cameron, 1978). Cohen et al (1979) have also reported a ten-fold increase in the concentration of deoxy-GTP in the lymphocytes of these patients. The biochemical consequences of PNP-deficiency are probably similar to those of ADA-deficiency except that deoxy-GTP is the likely toxic metabolite. Red cell transfusions have been successful in correcting the immunological defects of one PNP-deficient patient (Zegers et al, 1979).

1.3. CHRONIC LYMPHATIC LEUKEMIA

CLL is a malignant blood disease that occurs late in life (90% of the patients being above the age of 50) and the essential criterion for diagnosis is a sustained lymphocytosis in which the malignant cells are of monoclonal origin. Although T-cell CLL occasionally occurs, in the vast majority of patients there is an accumulation of B-lymphocytes. The absolute numbers of T-cells usually remains unchanged, although they are considerably diluted by the large excess of B-cells. At present there is no cure for CLL and treatment is largely symptomatic. In practice, many patients require no treatment at all.

CLL has been described as the accumulation of immunologically incompetent cells (Dameshek, 1967). In keeping with this view, both humoral and cellular immunity are impaired, and this secondary immunodeficiency

may manifest itself by a marked susceptibility to recurrent infections. The monoclonal origin of leukemic lymphocytes has been demonstrated by the observation that these cells express surface immunoglobulin with a single light chain type (Grey, Rabellino, & Pirofsky, 1971; Wilson & Nossal, 1971). The density of immunoglobulin on leukemic B-cells is generally lower than normal (Theil, Dormer & Eulitz, 1975) and they are thought to be arrested at an early stage of differentiation (Salmon & Seligmann,1974).

A method of clinical staging of the disease has been proposed which is based on the concept that CLL is a disease of progressive accumulation of non-functional lymphocytes (Rai et al, 1975). Attempts to correlate the 5'-N activity of CLL lymphocytes (see 1.1.8.) with the clinical stage of the disease (Kanter et al, 1979) and with the numbers of B- and T- lymphocytes (Quagliata et al, 1974; Kanter et al, 1979) have proved unsuccessful. Kanter et al did report an apparent correlation between the 5'-N activity of CLL lymphocytes and their *in vitro* blastogenic response to mitogens. They found that those patients with a normal 5'-N activity tended to show a better response to PHA than the patients with low or supranormal levels of 5'-N. However, the group of patients with normal 5'-N activity showed the least severe lymphocytosis, and would therefore be expected to contain a higher proportion of non-malignant cells.

CHAPTER 2

THE ROUTINE DETERMINATION OF THE 5'-NUCLEOTIDASE ACTIVITY OF CIRCULATING MONONUCLEAR CELLS : THE DEVELOPMENT AND APPLICATION OF A SUITABLE ASSAY.

2.1. INTRODUCTION

The assay used for the determination of 5'-N activity in these studies was based on the radiometric procedure employed by Avruch & Wallach (1971) for the study of rat fat cell 5'-N. Because this assay was not specifically designed for the determination of human lymphocyte ecto-5'-N activity, a reassessment of the optimum conditions was undertaken. The inclusion of β-glycerophosphate (Belfield & Goldberg, 1968) and L (+) tartrate (King & Jegatheesan, 1959) as inhibitors of non-specific phosphatase activity was considered in order to improve the specificity of the assay. The validity of objections raised against this type of assay (Chatterjee, Bhattacharya & Barlow, 1979) was also tested. Using a suitably modified assay, previous studies with CLL and hypogammaglobulinaemic patients were repeated. These results are discussed in relation to the apparent discrepancies between the results of Edwards et al (1978) and Webster et al (1978). The parents of some CV-H and X-H patients were also studied for evidence of a hereditary basis to the low 5'-N activity.

For convenience, the results in this chapter have been divided into two sections and discussed separately : the results of the investigation of the assay conditions are discussed before the presentation of the results obtained with the patients.

2.2. MATERIALS & METHODS

2.2.1. Materials

Chemicals were obtained from the sources listed.

Adenosine	SIGMA
2-(^{3}H)-Adenosine	RADIOCHEMICAL CENTRE, AMERSHAM
Adenosine 5'-monophosphate (AMP)	SIGMA
2-(^{3}H)-AMP	RADIOCHEMICAL CENTRE, AMERSHAM
p-Bis 2-(5-phenyloxazolyl)-benzene (POPOP)	NUCLEAR ENTERPRISES LTD.
2,5 Diphenyloxazole (PPO)	NUCLEAR ENTERPRISES LTD.
Ficoll 400	PHARMACIA
β -Glycerophosphate	SIGMA
Glycine	BDH
Isoton	COULTER ELECTRONICS LTD.
Sodium barbitone	BDH
Sodium cacodylate	BDH
Sodium potassium (+) tartrate	BDH
Triosil 440	NYEGAARD & CO., OSLO
Tris (hydroxymethyl) methylamine (tris)	BDH
Zaponin	COULTER ELECTRONICS LTD.

2.2.2. Isolation of peripheral blood mononuclear cells

Mononuclear cells were isolated from fresh defibrinated peripheral blood by Ficoll-Triosil density gradient separation (Böyum, 1968). Ficoll-Triosil, of specific gravity 1.077, was prepared from: 96 ml of 9% (wt/vol) Ficoll 400, 20 ml Triosil 440, and 20 ml H_2O. The solution was sterilised and stored in the dark at room temperature. Up to 15 ml of blood was layered onto 10 ml of Ficoll-Triosil, and centrifuged for 15 min at 1200g. Mononuclear cells were harvested from the gradient interface and washed (10 min at 200g) three times in isotonic 10 mM tris-buffered saline, pH 7.5 $_{20^oC}$ (TBS). The yield of mononuclear cells was usually between 0.5 - 1.0 x 10^6 cells per ml of normal blood.

2.2.3. Isolation of peripheral blood polymorphonuclear leukocytes

Fresh defibrinated blood was separated on a Ficoll-Triosil gradient as in 2.2.2., and the polymorphonuclear leukocytes recovered from the gradient pellet. The pellet was washed in TBS and then subjected to ammonium chloride lysis to remove the erythrocytes (Boyle, 1968). The washed cell pellet was resuspended in isotonic 0.14 M NH_4Cl_2 solution buffered with 0.017 M tris-HCl, pH 7.5_{20^oC}, which had been preheated to 37^oC. After 5 min incubation at 37^oC, the cells were washed twice in TBS at room temperature. A differential cell count was performed in order to determine the extent of contamination by mononuclear cells (usually less than 5%).

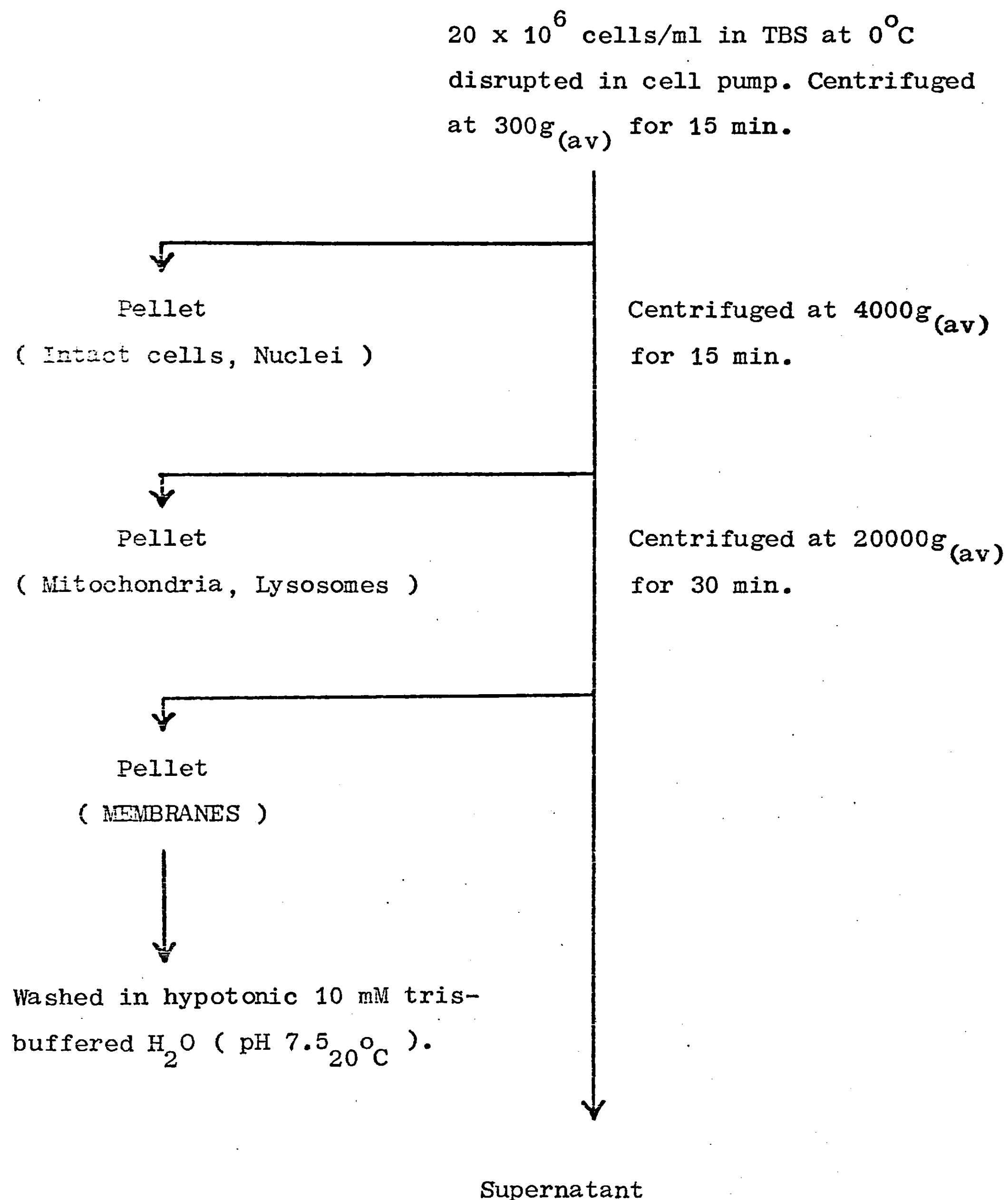
2.2.4. Isolation of tonsil lymphocyte plasma membranes

Human tonsils obtained within 2 hr of surgical removel provided a convenient source of large numbers of viable lymphocytes. Between $2 - 10 \times 10^8$ lymphocytes were obtained from each pair of tonsils. The tonsils were gently teased in calcium- and magnesium- free Dulbecco's phosphate-buffered saline (PBS) (Dulbecco & Vogt, 1954) and filtered through nylon gauze to remove clumps of tissue. Erythrocytes and some non-lymphoid cells were removed by Ficoll-Triosil density gradient centrifugation (2.2.2.). The mononuclear cells were washed three times in PBS and resuspended to about 2×10^7 cells/ml in ice-cold PBS. This cell-suspension usually contained greater than 95% lymphocytes, of which more than 90% were viable as shown by trypan blue dye exclusion.

The cells were disrupted at 0^oC by pumping the suspension through a cell disruption pump at 400 ml/hr (Wright, Edwards & Jones, 1974). This ruptured the cells, but not the subcellular particles, by forcing the suspension through a small oriface against a spring-loaded valve at a pressure of about 28 kg/cm^2. The disrupted cell suspension was then subjected to the differential centrifugal scheme shown in fig 3 (Allan & Crumpton, 1970). When the membranes were not used immediately, they were resuspended in 0.25 M sucrose buffered with 10 mM tris-HCl, pH 7.5 $_{20^oC}$, and stored at -70^oC.

Figure 3

SCHEME FOR THE ISOLATION OF MEMBRANES FROM LYMPHOCYTE CELL SUSPENSIONS



2.2.5. The 5'-nucleotidase assay

For the routine determination of 5'-N activity, the following stock solution was prepared and stored at -20°C until required:

0.04 mM AMP, with 0.4 μCi/ml tracer 2-(^{3}H)-AMP

0.12 mM β-glycerophosphate

20 mM $MgCl_2$

200 mM glycine-NaOH buffer, pH 8.5

Between 10^5 - 10^6 cells in TBS were mixed with an equal volume of the stock substrate solution, and incubated at 37°C for 20 min. The reaction was terminated by transferring the assay tubes to an ice bath and adding 0.3 ml of 0.125 M $ZnSO_4$ solution (which inhibits 5'-N activity). Unreacted AMP was precipitated by the addition of 1.0 ml of $Ba(OH)_2$ solution, and removed by centrifugation at 2000g for 5 min. One ml aliquots of the supernatents were counted for tritium in 9.0 ml of xylene-Triton scintillation fluid (15 g PPO, 0.3 g POPOP, 2.5 l xylene, 1.25 l Triton X-100, 375 ml methanol).

All determinations were performed in duplicate, and background counts were determined from blanks in which 0.5 ml of TBS was added in place of the cell suspension. Standards were prepared by diluting 0.5 ml of the stock substrate solution with 1.8 ml of TBS, and counting 1.0 ml aliquots. The enzyme activity was expressed in units of : nmol AMP hydrolysed/hr/10^6 cells. The following expression was used to determine the 5'-N activity:

$$\text{5'-N activity} = \frac{(X - B).\ 6}{(S - B).\ N} \times 10^5$$

where : X = c.p.m. of sample

B = c.p.m. of blank

S = c.p.m. of standard

N = number of cells present in sample

The cell concentrations for the assay were determined electronically using a Coulter Counter (see 2.2.6.)

2.2.6. Determination of cell concentration using the Coulter Counter (Model Dn)

The Coulter Counter works on the principle that cells suspended in isotonic saline will displace their own volume of electrolyte when passing through an electric field, and thus modulate the electric current passing between the electrodes. The resulting electrical impulse is amplified and applied to a digital counter. The electronic counting of cells is quicker and more reproducible than visual enumeration using a haemocytometer. In practice, 0.5 ml of the cell suspension is drawn through a 50 micron diameter orifice in an insulating wall between two electrodes. Threshold controls are set to exclude from count the small pulses due to cell fragments, platelets, etc. Automatic compensation is made for the error caused by the simultaneous passage of two or more cells through the orifice.

When counting a suspension of leukocytes, 0.1 - 1.5 x 10^6 cells were diluted to 10 ml with 'Isoton' (a dust-free commercial isotonic electrolyte solution) and three drops of 'Zaponin' (an acetate-based lysing solution) were added to lyse contaminating erythrocytes. The optimum threshold was determined by calibration with peripheral blood lymphocytes, and was checked periodically.

2.2.7. Statistical analysis

In any one group of observations, the standard deviation (s.d.) is given by :

$$\text{s.d.} = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}}$$

where

x = A single observation

n = The number of observations in a group

$\bar{x}$ = The mean of n observations

The standard error of the mean (SEM) is given by :

$$\text{SEM} = \frac{\text{s.d.}}{\sqrt{n}}$$

In population studies, there is a 95% probability that any given value from a population will fall within 2 x s.d. of the mean value. In this thesis, the term 'normal range' refers to the 95% probability range.

Tests of significance

(a) Student's t-test:

When testing whether the mean values of two populations are significantly different, Student's t-value is determined as:

$$t = \frac{m_1 - m_2}{S\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

where

m_1 and m_2 = the mean values of each of the two populations

n_1 and n_2 = the number of components of the two populations

$$S = \sqrt{\frac{a^2 (n_1 - 1) + b^2 (n_2 - 1)}{n_1 + n_2 - 2}}$$

where (a) and (b) are the s.d. for m_1 and m_2 respectively.

The probability (p) that a difference between the two means occurs by chance will be a function of t and the number of degrees of freedom ($n_1 + n_2 - 2$). The p-value is readily obtained from t-distribution tables, and where $p < 0.05$ the two means are considered to be significantly different. When testing whether the mean value, m, of n observations is significantly different from a given value, v, the following expression is used to determine Student's t-value:

$$t = \frac{m - v}{(SEM)}$$

(b) Student's paired t-test:

This analysis is applicable when dealing with two paired groups of observations. Thus:

$$x_1 - x_2 = d$$

where x_1 and x_2 are paired observations, and d is the difference between them.

$S(\bar{d})$ is the standard deviation of the mean difference for n pairs of observations, and is given by:

$$S(\bar{d}) = \sqrt{\frac{\sum (d - \bar{d})^2}{n - 1}}$$

where $\bar{d} = \frac{\Sigma d}{n}$

The value of Student's t-test is given by: $t = \frac{\bar{d}}{S(\bar{d})}$

2.3. INVESTIGATION OF THE 5'-NUCLEOTIDASE ASSAY CONDITIONS

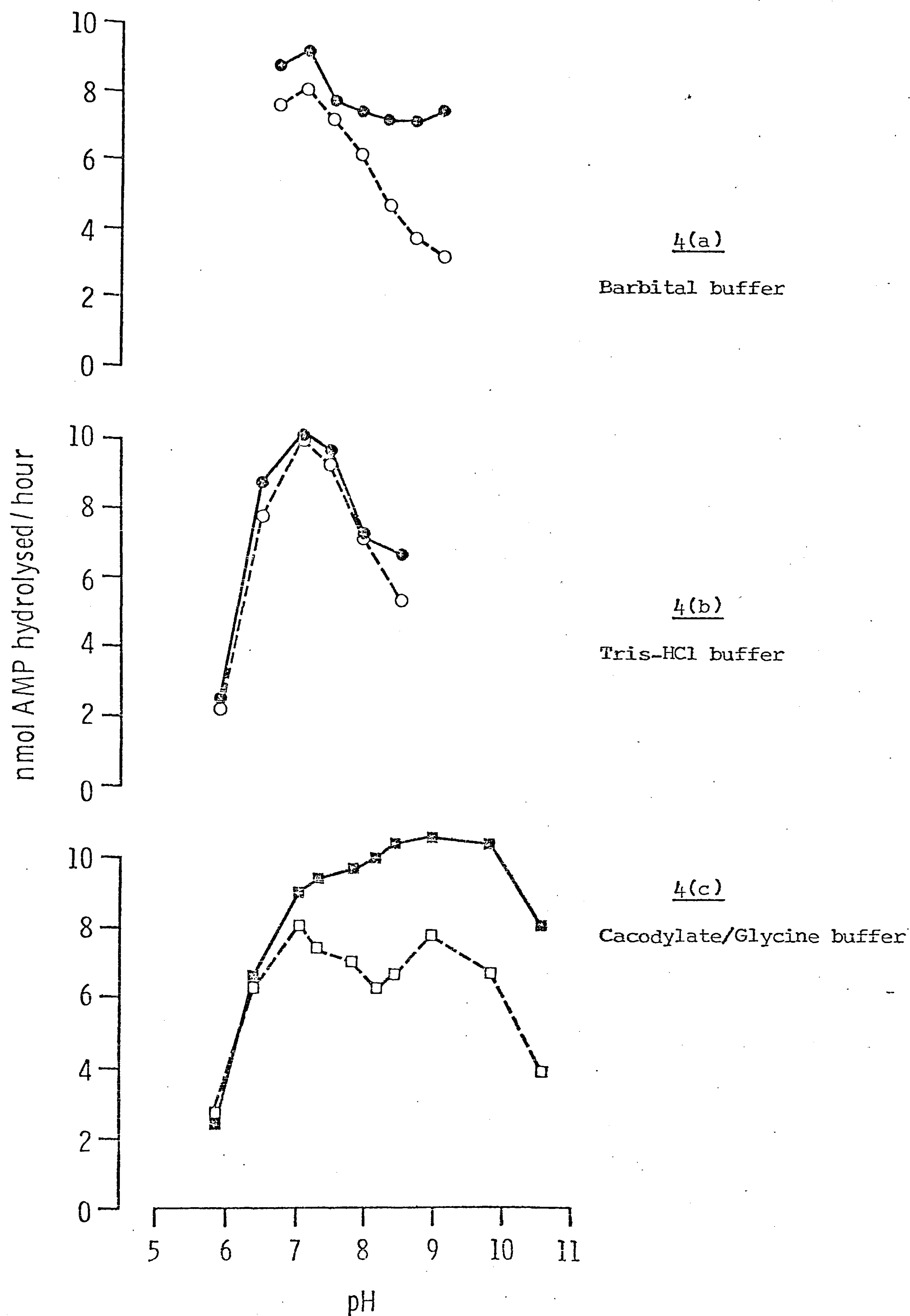
2.3.1. RESULTS

2.3.1.1. The pH optimum of human lymphocyte 5'-nucleotidase

Tonsil lymphocyte plasma membranes were used as a convenient source of 5'-N activity. Different buffers were used to obtain pH profiles over three different ranges. Apart from the buffer, the standard substrate mixture (see 2.2.5.) was used for the determination of 5'-N activity. β-glycerophosphate was retained in the mixture to inhibit any possible non-specific phosphatase activity (see 2.3.1.2.). The pH of the reaction mixtures was determined at 37°C.

Barbital buffer (0.05 M) was used over the pH range 6.8 - 9.2 (fig 4a). In this system, optimum activity was observed at around pH 7.2; both in the absence and presence of 10 mM $MgCl_2$. In the absence of $MgCl_2$ there was a sharp loss of 5'-N activity above pH 7.5; but in the presence of this salt, a second optimum appeared above pH 9.0. Similar results were obtained with Tris-HCl buffer over the pH range 5.3 - 8.6 (fig 4b), except that the activation observed with $MgCl_2$ above pH 7.5 was less pronounced with this buffer. A dual buffer system employing 0.05 M sodium cacodylate and 0.05 M glycine was used to obtain a continuous profile over the pH range 5.9 - 10.6 (fig 4c). This system buffered well between 5.9 - 7.7 and 8.1 - 10.6 when titrated with HCl and NaOH. In the absence of $MgCl_2$, two pH optima were obtained : at about 7.1 and 9.0. In the presence of this salt, a broad plateau of optimum activity was observed between pH 8.4 - 9.8.

Figure 4 pH PROFILES OF 5'-NUCLEOTIDASE ACTIVITY



The unbroken lines illustrate the activity in the presence of 10 mM $MgCl_2$,and the dashed lines denote the absence of this salt.

The Tris-HCl buffer system was found to produce undesirably high background counts. Typically, the blanks gave counts of between 8 - 10% of the standard. This compared unfavourably with the glycine and cacodylate buffers which gave blank counts of less than 1% of the standard, and with the barbital buffer which gave a blank count of 1-2% of the standard.

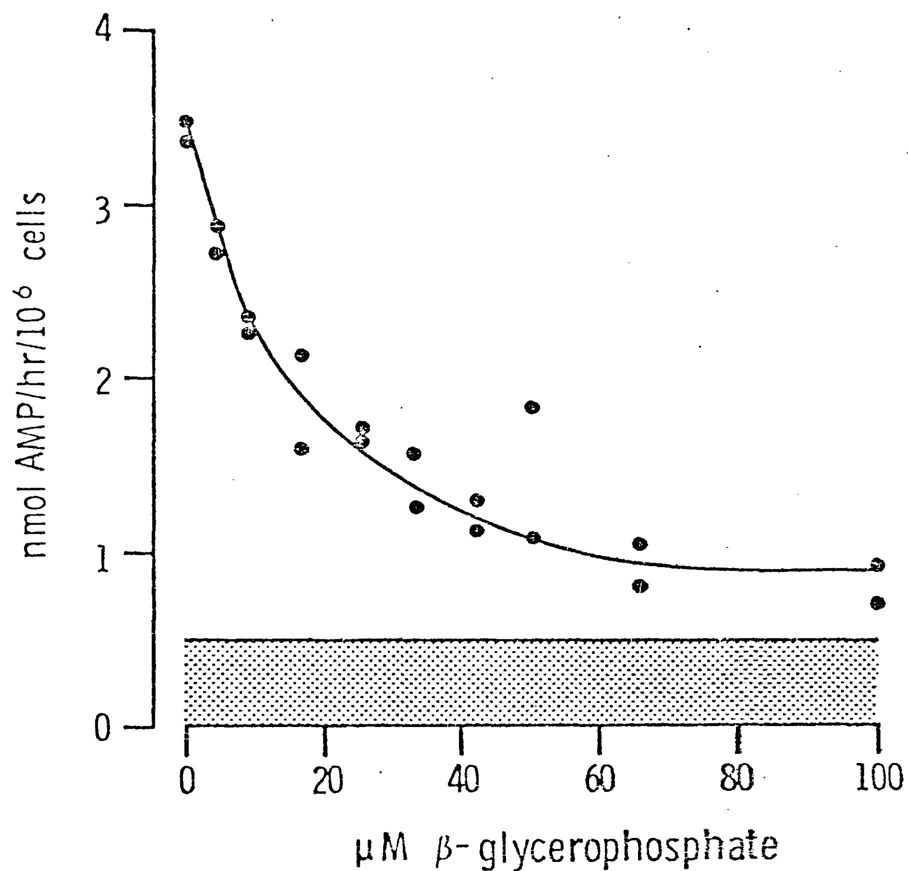
2.3.1.2. Inhibition of non-specific phosphatase activity

Intact human peripheral blood polymorphonuclear leukocytes were used as a source of alkaline phosphatase activity (Park, 1970). The formation of adenosine from AMP was followed in the presence of 0 - 100 μM β-glycerophosphate using the basic 5'-N assay described in 2.2.5. Contamination of the polymorphonuclear leukocyte preparations by mononuclear cells was determined by staining the cells with crystal violet and performing a differential cell count by light microscopy. In the experiment illustrated here (fig 5) there was a 2.3% contamination by mononuclear cells, and the contribution of the 5'-N activity of these cells to the breakdown of AMP by the polymorphonuclear cell preparation is indicated. About 85% of the phosphatase activity of the polymorphonuclear cells was inhibited by 60 - 100 μm β -glycerophosphate.

Sodium potassium tartrate, an inhibitor of acid phosphatase activity, appeared to interfere with the 5'-N assay. Table 3 summarizes the effect of tartrate on the 5'-N activity of peripheral blood mononuclear cells. The presence of 10 mM tartrate (the concentration

Figure 5

THE INHIBITION OF ALKALINE PHOSPHATASE BY β-GLYCEROPHOSPHATE



The shaded area represents the amount of 5'-nucleotidase activity due to contaminating mononuclear cells in the polymorphonuclear leukocyte preparation.

usually employed to inhibit acid phosphatase) increased the background counts of the assay by more than five-fold.

TABLE 3

THE EFFECT OF SODIUM POTASSIUM TARTRATE ON THE APPARENT 5'-NUCLEOTIDASE ACTIVITY OF PERIPHERAL BLOOD MONONUCLEAR CELLS

Buffer used in 5'-N assay	5'-N activity (nmol/hr/10^6 cells)		
	Concentration of tartrate (mM)		
	0	1	10
Tris-HCl, pH 5.2	0.0	0.0	5.8
Tris-HCl, pH 8.5	7.0	7.6	8.3
Glycine-NaOH, pH 8.5	11.0	11.4	13.0

2.3.1.3. Properties of the 5'-N assay

Intact viable tonsil lymphocytes (prepared as in 2.2.4.) were used to characterize the 5'-N assay. Fig. 6 shows the results from a typical experiment in which the percentage hydrolysis of substrate after 20 min reaction was determined for a range of cell concentrations. The rate of reaction was found to be linear up to about 35% hydrolysis. The rate of reaction was linear for at least 30 min provided that the substrate hydrolysis did not exceed 35% (fig 7).

Figure 6

RELATIONSHIP BETWEEN THE 5'-NUCLEOTIDASE ACTIVITY AND ENZYME CONCENTRATION

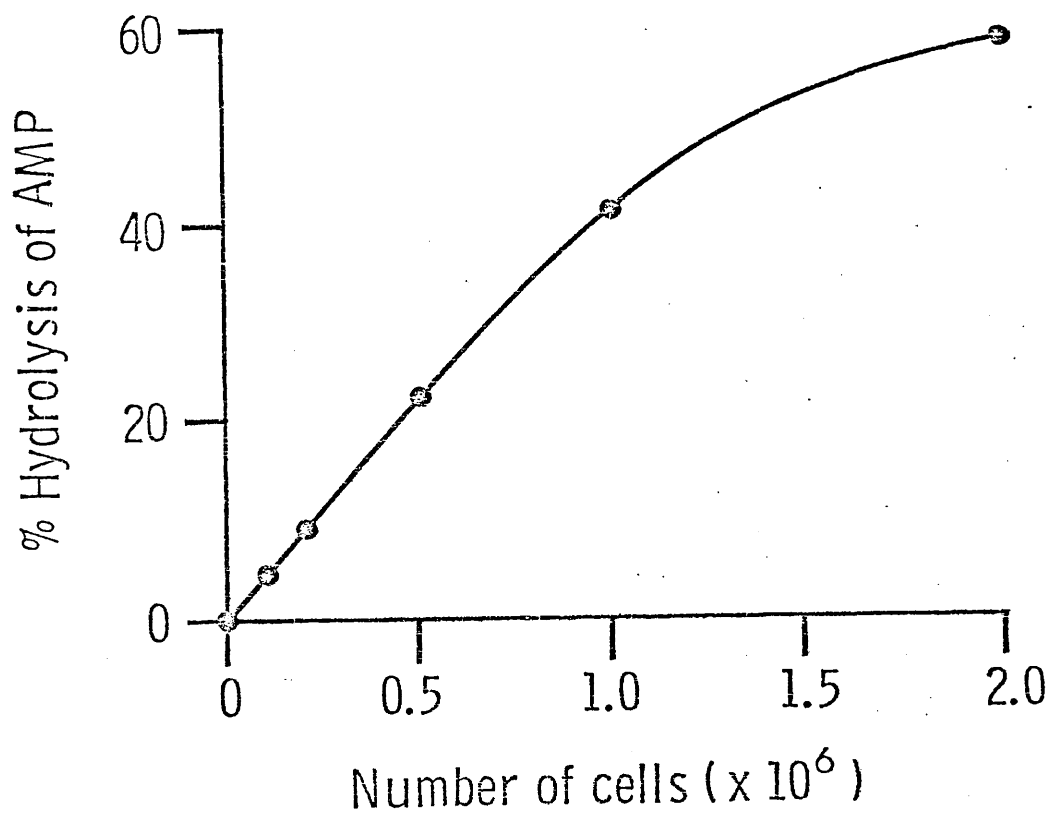
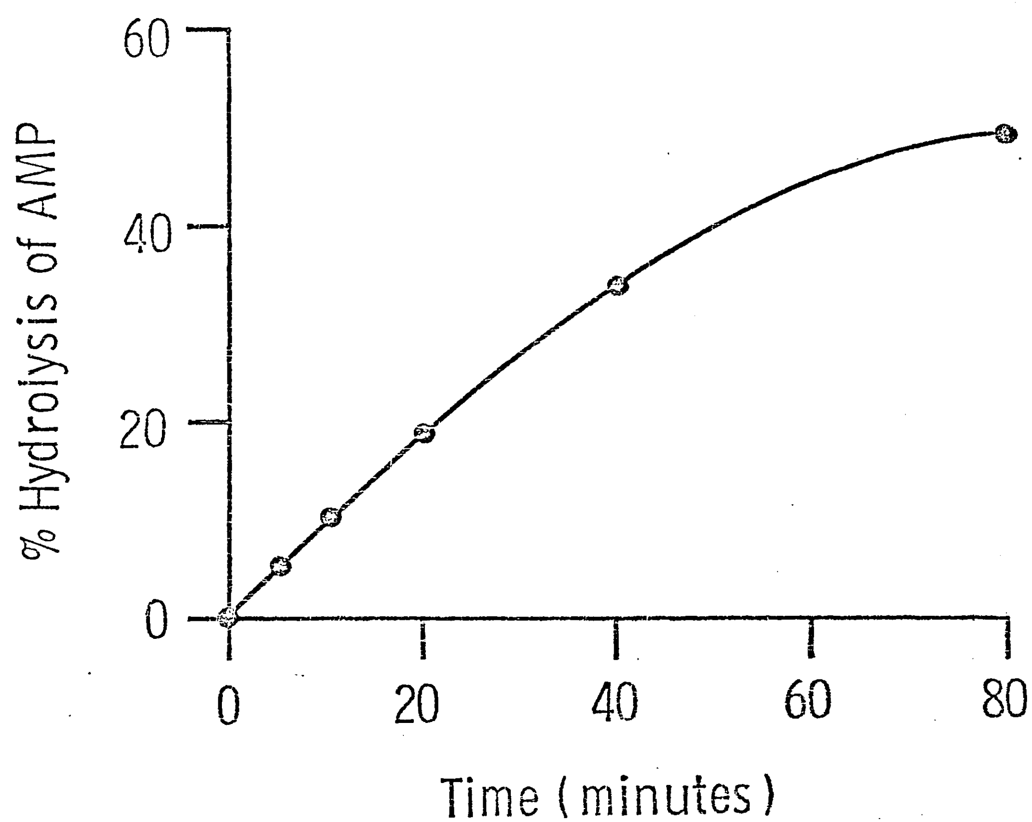


Figure 7

RELATIONSHIP BETWEEN THE 5'-NUCLEOTIDASE ACTIVITY AND TIME OF REACTION



In order to assess the extent of any apparent 'reverse 5'-N' activity that might result from the further metabolism of adenosine, the assay was performed with adenosine in place of the AMP substrate. (2-^{3}H)-adenosine label was used at a concentration of 0.2 µCi/ml to follow the reaction. The apparent reverse 5'-N reaction at 20 µM adenosine (which is the concentration of AMP used in the 5'-N assay) was 0.84 nmol/hr/10^6 cells in a lymphocyte preparation that gave a 5'-N activity of 24.0 nmol/hr/10^6 cells (fig 8). The underestimation of 5'-N activity was therefore less than 3.5%.

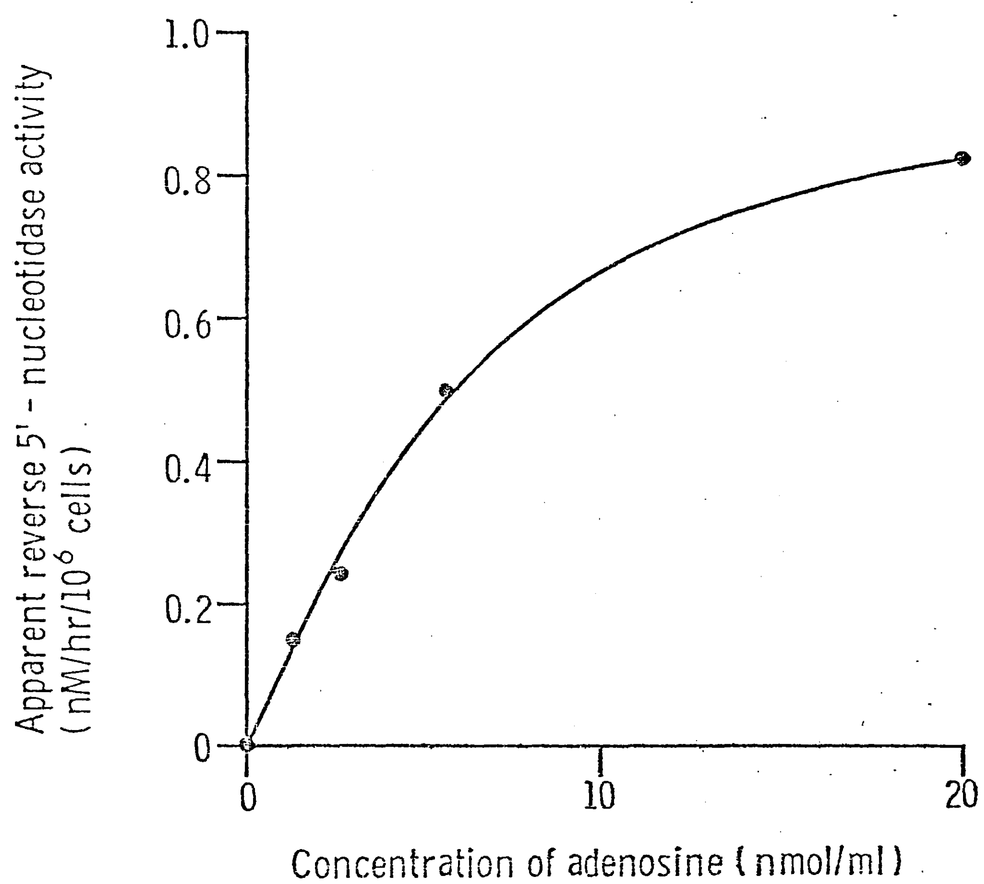
2.3.2. DISCUSSION

The effects of Mg^{++} ions and glycine buffer on the pH profile of human lymphocyte plasma membrane 5'-N were similar to those observed with bull semen plasma 5'-N (Bodansky & Schwartz, 1963; Levin & Bodansky, 1966). The properties of the human lymphocyte enzyme therefore resemble those of the liver plasma membrane enzyme from various species rather than the pig lymphocyte plasma membrane enzyme (see 1.1.4.).

The differences between human and pig lymphocyte membrane 5'-N emphasize the need for checking the suitability of the assay conditions. The results obtained here with Tris-HCl buffer (fig 4b) show that the original conditions employed by Avruch & Wallach (1971) (Tris-HCl buffer, pH 8.5, with Mg^{++}) are sub-optimal for human lymphocyte 5'-N. Furthermore, the background counts obtained with this buffer are higher than those obtained with the other buffers studied. The use of Tris-HCl buffer therefore results in an unnecessary loss of sensitivity.

Figure 8

APPARENT REVERSE 5'-NUCLEOTIDASE ACTIVITY



For the routine measurement of 5'-N activity, Glycine-NaOH buffer, pH 8.5, was used with 10 mM $MgCl_2$. At this pH, the inclusion of an inhibitor of acid phosphatase activity was considered unnecessary. However, β-glycerophosphate was included as a precaution against possible alkaline phosphatase activity; although in practice, human circulating mononuclear cells were found to have a negligible non-specific phosphatase activity under these assay conditions compared to their 5'-N activity. The substrate concentration employed (20 μM) is about double the value of the apparent Michaelis constant (see 3.3.2.). Although the modified assay showed a linear release of adenosine up to about 35% hydrolysis, this is considerably less than the 80% reported for the original assay with rat fat cell membranes (Avruch & Wallach, 1971). However, the working range of the assay was more than adequate.

Chatterjee, Bhattacharya & Barlow (1979) have recently criticised this type of assay in which $ZnSO_4$ and $Ba(OH)_2$ are used to quantitatively precipitate AMP. They suggest that because adenine, guanine, guanosine, hypoxanthine and inosine are also precipitated under these conditions, the presence of ADA (adenosine deaminase) would lead to an under-estimation of the 5'-N activity. However, the results shown in fig 8 suggest that this criticism is not valid for the assay of lymphocyte 5'-N employed in this study. Presumably the activities of ADA and adenosine kinase are small compared to the 5'-N activity under these assay conditions.

2.4. THE 5'-NUCLEOTIDASE ACTIVITY OF CIRCULATING MONONUCLEAR CELLS FROM NORMAL INDIVIDUALS AND PATIENTS

2.4.1. PATIENTS STUDIED

2.4.1.1. Patients with X-linked hypogammaglobulinaemia

Ten patients, aged 9 - 28 yrs, were studied. All had affected male relatives and were receiving weekly gammaglobulin injections. The percentage of B-lymphocytes in the circulating mononuclear cells of these patients was less than 1% when tested either by fluorescent antibody or by rosetting techniques (see chapter 3). The mean serum IgG concentration was 2.8g/l (range, 0.5 - 4.6) and the IgA and IgM levels were less than 0.1g/l.

The normal '95% range' found for serum immunoglobulin concentrations in the immune-deficiency referral laboratory at the Clinical Research Centre is as follows: IgG, 5.9 - 17.2g/l; IgA, 0.5 - 4.0g/l; IgM, 0.6 - 2.0g/l.

2.4.1.2. Patients with 'common variable' hypogammaglobulinaemia

Twenty six patients with CV-H, aged 7 -51 yrs, were studied. All but four developed their disease after the age of sixteen. The mean serum IgG concentration in these patients was 2.5 g/l (range 0.7 - 6.4); one patient, P.M., whose IgG level of 6.4 was just within the normal range, had undetectable levels of IgA and IgM by the radial immunodiffusion technique. All the patients had less than 0.1 g/l serum IgA, and 60% had less than 0.1 g/l serum IgM; the range of serum

IgM concentrations for all the CV-H patients being from less than 0.1 g/l to 0.6 g/l. These patients were receiving regular gamma-globulin replacement therapy.

2.4.1.3. Patients with selective IgA-deficiency (IgA^- patients)

Six patients, aged 7 - 33 yrs, were studied. All had less than 0.1 g/l serum IgA and normal levels of IgM and IgG. All the patients suffered recurrent chest infections. Patient N.G. had Wegener's granuloma, and his sister had died with severe hypogamma-globulinaemia and neutropenia.

2.4.1.4. Patients with Chronic Lymphatic Leukemia

Thirteen patients with CLL were studied in this thesis. Their mean age was 62 yrs (range 48 - 70) and the number of patients in each of the clinical stages of the disease as defined by Rai et al (1975) were: Stage 0, 2 patients; Stage I, 1; Stage II, 5; Stage III, 4; Stage IV, 1. Patient T.R., was unusual in that he had a grossly enlarged liver (with an apparently normal histology on biopsy) without any enlargement of the spleen or lymph nodes. Also, the morphology of the leukemic cells from this patient, together with the density of surface immunoglobulin, were suggestive of a more differentiated cell than is characteristic of CLL. This patient showed some characteristics of both CLL and prolymphocytic leukemia (Catovsky, Costello, O'Brien & Cherchi, 1979). Four patients were receiving treatment at the time of study: two were receiving chlorambucil, one was receiving allopurinol with

chlorambucil, and the fourth was receiving cyclophosphamide, vincristine, and prednisolone.

2.4.2. RESULTS

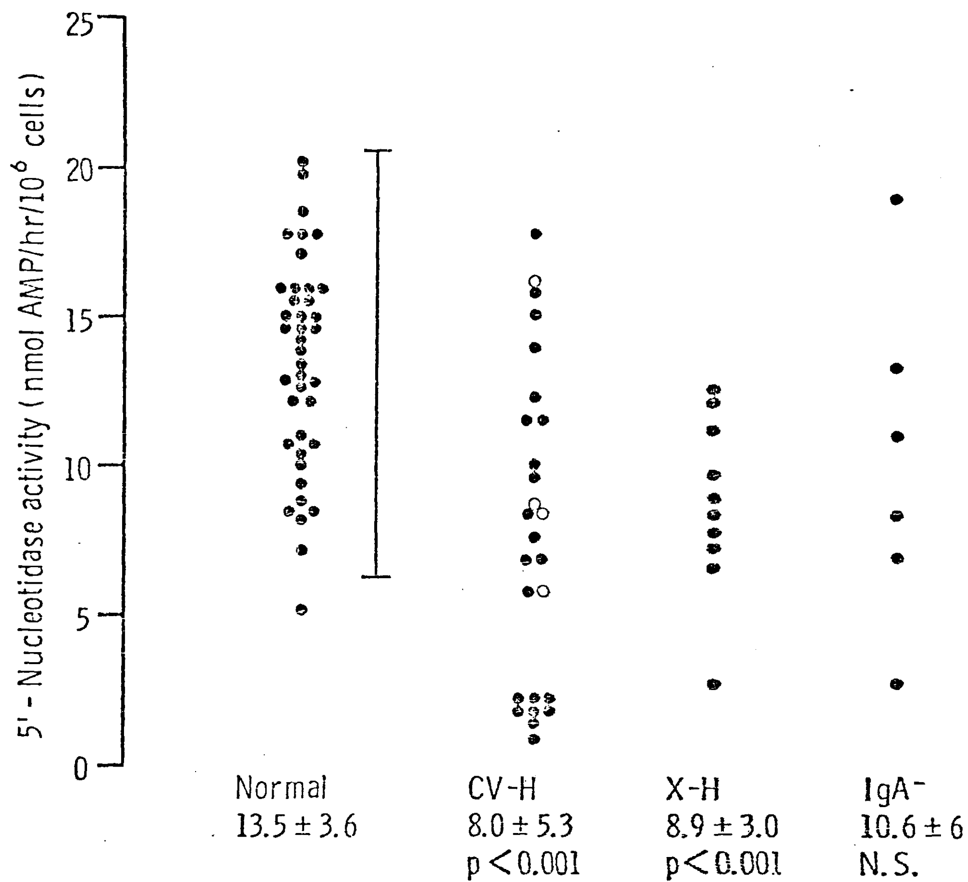
2.4.2.1. The 5'-nucleotidase activity of circulating mononuclear cells from patients with CLL or hypogammaglobulinaemia

The 5'-N activities of circulating mononuclear cells from 26 CV-H patients, 10 X-H patients, and 40 healthy subjects, are compared in fig 9. The ages of the patients ranged from 7 - 51 yrs, and the controls ranged from 19 - 52 yrs. Ten of the 26 CV-H patients had a 5'-N activity that was below the normal range. The X-H patients showed a similar distribution of 5'-N activities to the CV-H patients, although only one was below the normal range. Only six patients with IgA-deficiency were available for study, and the results were inconclusive. The mean activities of the CV-H and X-H patients were both significantly lower than normal (Student's t-test, $p < 0.001$), but the mean activity of the IgA-deficient patients was not significantly different from either the mean normal activity or the mean activity of CV-H patients.

The 5'-N activities of 10 patients with CLL were compared with those of 20 normals of ages 40 - 82 yrs (fig 10). Eight patients were below the normal range, and one (patient T.R.) had an activity that was about twelve times higher than the normal mean. The mean 5'-N activity of the 20 normal donors above the age of 40 was significantly less (Student's t-test, $p < 0.001$) than the mean activity of the 40 donors between the age of 19 - 52.

Figure 9

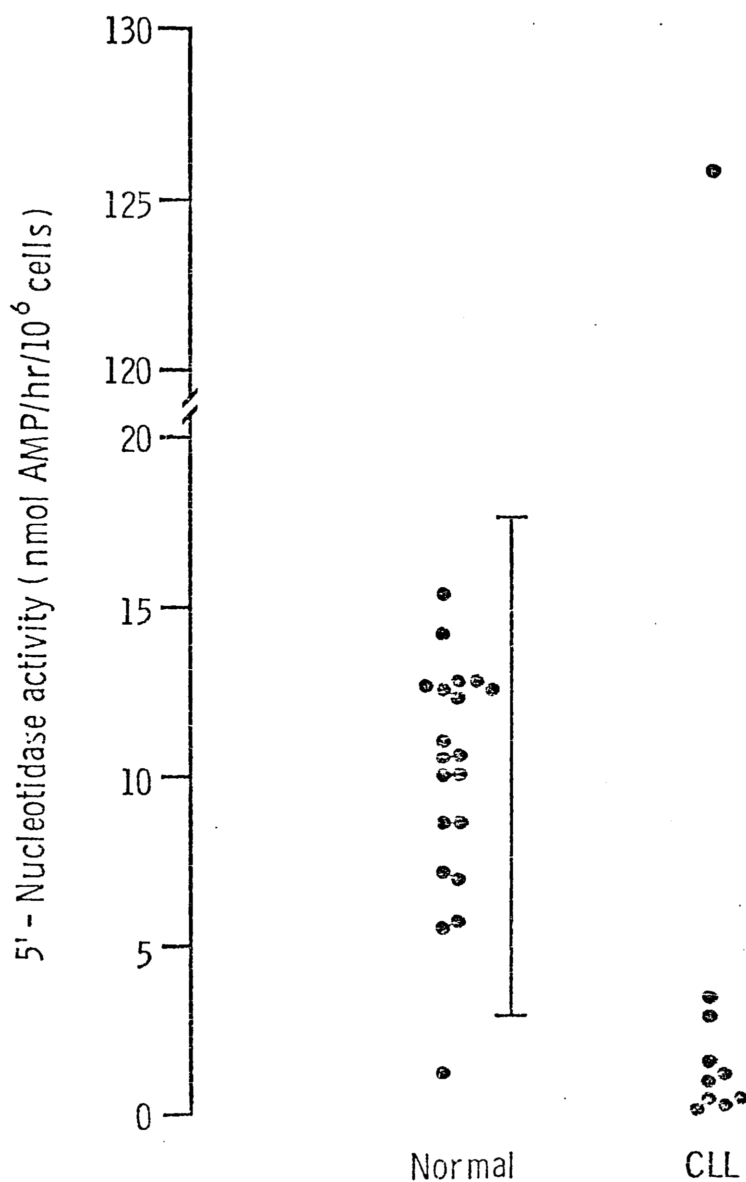
5'-NUCLEOTIDASE OF CIRCULATING MONONUCLEAR CELLS FROM PATIENTS WITH HYPOGAMMAGLOBULINAEMIA



The ages of the controls ranged from 19-52 yrs. The vertical bar represents the 'normal range' of 5'-nucleotidase activity (mean ± 2 s.d.). The open circles denote 'childhood-onset' CV-H patients.

Figure 10

5'-NUCLEOTIDASE OF CIRCULATING MONONUCLEAR CELLS FROM PATIENTS WITH CLL



The ages of the controls ranged from 40-82 yrs, and the vertical bar represents the 'normal range' of 5'-nucleotidase activity.

2.4.2.2. The 5'-nucleotidase activity of the circulating mononuclear cells from the parents of some patients with hypogammaglobulinaemia

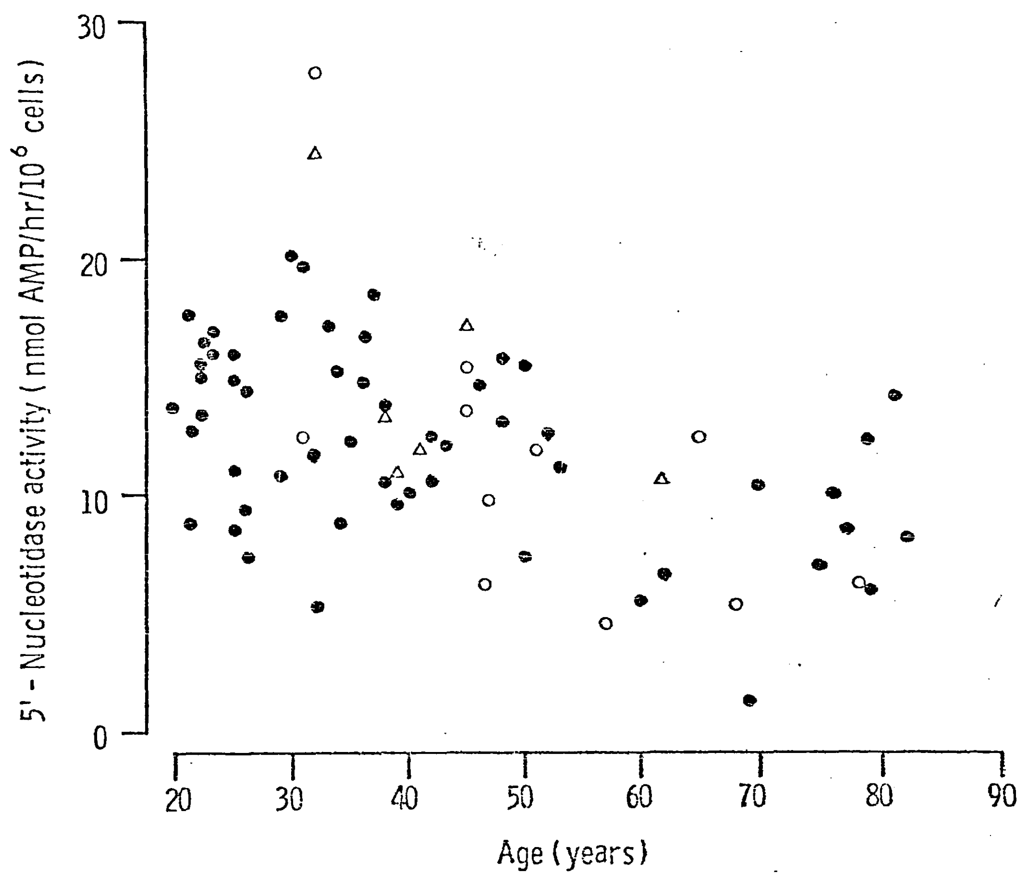
The 5'-N activities of circulating mononuclear cells from eleven parents (3 fathers and 8 mothers) of patients with CV-H, and from six mothers of patients with X-H, are compared in fig 11. The patients in question all had a 5'-N activity that was below the normal mean. Because of the decrease in the 5'-N activity in older subjects, the results are expressed in the form of a scatter-plot. The parents appeared to have a normal activity for their age.

2.4.3. DISCUSSION

Using the modified assay described earlier, reports in the literature of low 5'-N activity in the majority of patients with CLL have been confirmed. Patient T.R., who had a supranormal level of 5'-N activity presented with unusual clinical and morphological features (see 2.4.1.4.), but other CLL patients with supranormal 5'-N activity have been reported previously (Quagliata et al, 1974; LaMantia et al, 1977; Kanter et al, 1979). The original observation of low 5'-N activity in many patients with CV-H (Johnson et al, 1977) has also been confirmed. The apparently normal 5'-N activities reported in the six patients studied by Edwards et al (1978) may be due to the limited number of patients studied. In addition, most of their patients had an onset of symptoms in the pediatric age-group (personal communication, Irving H. Fox) and were therefore clearly different from the 'adult onset'

Figure 11

5'-NUCLEOTIDASE OF CIRCULATING MONONUCLEAR CELLS FROM THE PARENTS OF PATIENTS WITH HYPOGAMMAGLOBULINAEMIA



● Normal controls

○ Parents of patients with CV-H

△ Mothers of patients with X-H

patients studied by Johnson et al (1977); although Webster et al, (1978) showed that 'childhood onset' and 'adult onset' CV-H patients had similar 5'-N activities. The limited number of patients studied also appears to be the cause of the conflicting results obtained with IgA-deficient patients. If the results obtained by Webster et al (1978) and Edwards et al (1978) are considered together with the results obtained here, it appears likely that these patients may show a similar distribution of 5'-N activities to the patients with CV-H. The results obtained with X-H patients in this thesis (see also 3.3.1.) suggest that both the very low 5'-N activities reported by Edwards et al (1978) and the low-normal values reported by Webster et al (1978) are found in these patients, and that the scatter of 5'-N levels is greater than was previously thought.

In view of the lack of convincing evidence for a hereditary defect in the majority of CV-H patients, it was perhaps not surprising that the parents of these patients had a normal 5'-N activity (fig 11). The heterozygote mothers of X-H patients also had normal levels of 5'-N activity. Thus, in this limited investigation, no evidence was found to suggest that the low levels of 5'-N were inherited.

CHAPTER 3

THE NATURE OF THE 5'-NUCLEOTIDASE DEFICIENCY IN HYPOGAMMAGLOBULINAEMIA

3.1. INTRODUCTION

The apparently isolated biochemical abnormality of low 5'-N activity in some X-H and CV-H patients (Webster et al, 1978) provides us with a potential tool for clarifying the function of the enzyme. Elucidation of the cause and consequences of the low enzyme levels in these patients should hopefully provide vital clues as to the function of the enzyme in lymphocytes.

There are a number of possible explanations for the reduced 5'-N activity in the patients. At the biochemical level, the enzyme molecule may be defective or under the control of a regulatory factor. Alternatively, the rate of synthesis or degradation of the enzyme may be altered; resulting in fewer enzyme molecules per cell. At the cellular level, there is the possibility that a population of cells particularly rich in 5'-N activity may be absent. Since patients with X-H lack circulating B-cells, and when these cells are present in CV-H patients they are functionally defective, these cells appear to be likely candidates for the cause of the low 5'-N levels at the cellular level. However, B-cells represent only a minor percentage (4-26%) of peripheral blood mononuclear cells (Gmelig-Meyling & Ballieux, 1977) and previous workers have been unable to detect a difference between the 5'-N activity of B- and T- cells from normal individuals (see 1.1.8.).

The experiments in this section were designed to investigate the cause of the reduced levels of 5'-N in CV-H and X-H patients. In addition to kinetic studies, the situation regarding the level of 5'-N on B- and T-cells was reinvestigated using recently developed improved procedures for isolating these cells. The results obtained with the mononuclear cells of patients were compared with those of cord blood mononuclear cells which also have less 5'-N activity than the cells from healthy adults (see 1.1.8.).

3.2. MATERIALS & METHODS

In order to avoid unnecessary repetition, only the materials and methods not already described in chapter 2 are given here. Sterile precautions were taken where appropriate.

3.2.1. Materials

Chemicals and other materials were obtained from the sources listed :

Material	Source
Antibody solutions	see 3.2.3.
Cells: Ox erythrocytes	WATFORD SLAUGHTER HOUSE
Sheep erythrocytes	TISSUE CULTURE SERVICES
Chromic chloride	BDH
Foetal calf serum	GIBCO BIOCULT
L-Glutamine	BDH
Heparin	PAINES & BYRNE LTD
Neuraminidase	BEHRINGWERKE AG, MARBURG
Penicillin	GLAXO
R.P.M.I. 1640 medium, buffered with either bicarbonate or hepes	FLOW LABORATORIES, SCOTLAND
Sodium azide	BDH
Sodium metrizoate	NYEGAARD & CO., OSLO
Streptomycin	GLAXO
Triton X-100	BDH
Bijou culture bottles (plastic)	STERILIN LTD

3.2.2. Patients studied

Fourteen patients with CV-H were studied (ages 19-51 yrs), eight of whom were included in previous study. Eight patients with X-H were studied (ages 9-28 yrs), three of whom were included in the previous study. The serum immunoglobulin levels and the treatment of these patients were similar to those described in chapter 2. The mean age of the normal adults used as controls was 31 yrs (range 21-68 yrs). Cord blood was obtained from placentae following normal full-term deliveries.

3.2.3. Goat antibodies to human immunoglobulins

Antibody solutions were kindly provided by Dr. T.A.E. Platts-Mills (Dept. Immunological Medicine, C.R.C.). The preparation of such large quantities of specific antibodies from commercially-available anti-sera would have been unnecessarily expensive. Goat antisera to human immunoglobulin were raised, and the antibodies purified as described by deGast & Platts-Mills (1979). Briefly, anti-IgM antiserum was passed through a human IgG immunoabsorbent column to remove antibodies to IgG and light chains. The anti-IgM antibodies were then purified by specific adsorption to a human IgM column. This antibody solution did not show any anti-IgA, anti-IgG, or anti-light chain activity. An anti-IgG antiserum was purified by specific adsorption to a human IgG column. This antibody solution showed a strong anti-IgG and anti-kappa light chain activity. The anti-IgM and 'anti-IgG' antibody solutions were mixed 1:1 to give an anti-Ig antibody solution that was used for the isolation of surface-Ig positive (sIg^+) cells.

3.2.4. Isolation of peripheral blood mononuclear cell populations

Since relatively large numbers of cells were required for these experiments, heparinized blood was used in preference to defibrinated blood. Heparinized blood generally yielded about twice as many mononuclear cells as defibrinated blood, and the presence of platelets did not interfere with the isolation procedures or the 5'-N assay. Before separating the mononuclear cells on a Ficoll-Triosil gradient (2.2.2.), the blood sample was diluted with an equal volume of R.P.M.I. (hepes) medium: otherwise the cell separation tended to be poor due to 'overloading' of the gradient. Sub-populations of the mononuclear cells were isolated according to the scheme outlined in fig. 12.

3.2.4.1. Separation of rosetting and non-rosetting lymphocytes by density-gradient centrifugation on Ficoll-Metrizoate (specific gravity, 1.090)

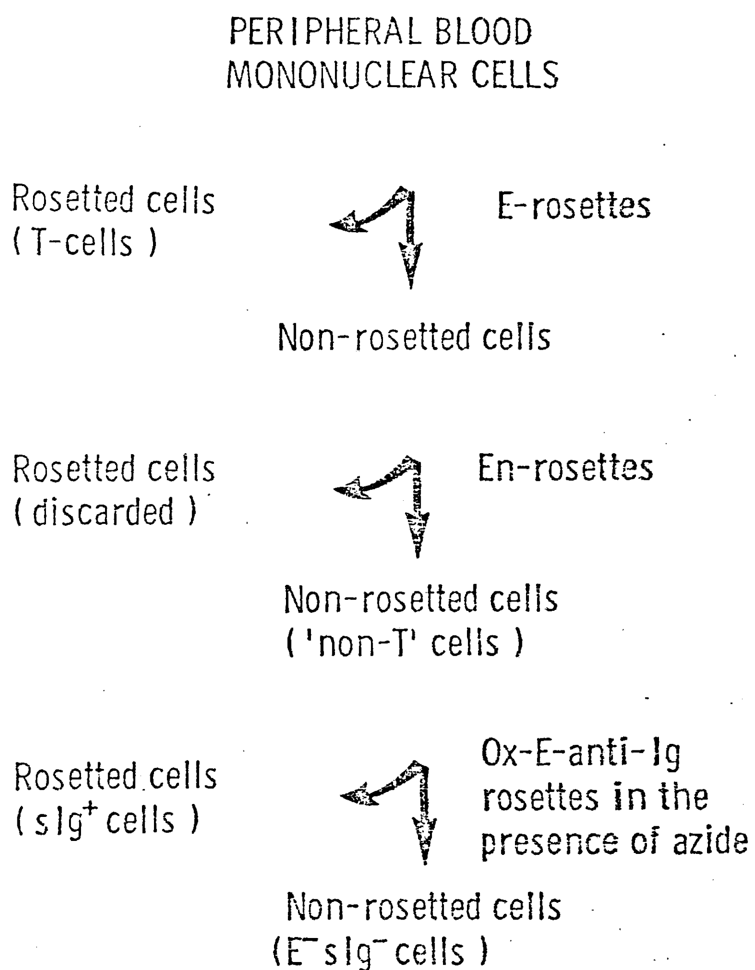
The use of Ficoll-Metrizoate to separate rosetted cells was described by Parish & Hayward (1974b). The separation medium was prepared from 12 parts of 14% Ficoll 400 (wt/vol) and 5 parts of 32% sodium metrizoate. The rosetted cell suspension was layered onto this Ficoll-Metrizoate mixture, and centrifuged at room temperature for 30 min at 1200g. The best separations were obtained by attaining the 1200g force within 20s by rapid initial acceleration. Red cells and rosetted cells were recovered from the pellet, and the non-rosetted cells were recovered from the gradient interface. The pellet was washed once in PBS before subjecting to ammonium chloride lysis (see 2.2.3.) to remove the erythrocytes.

3.2.4.2. Separation of T-cells by E-rosetting

The procedure for isolating T-cells was essentially that of Gmelig-Meyling & Ballieux (1977), which utilizes the property of these cells to

Figure 12

SCHEME FOR THE ISOLATION OF MONONUCLEAR CELL SUBPOPULATIONS



form spontaneous rosettes with sheep erythrocytes (E-rosettes). Sheep erythrocytes, which were less than one week old and stored in Alsever's solution, were washed three times in PBS immediately before use. Foetal calf serum (FCS) which had been inactivated by heating to 56°C for 30 min, was absorbed for 2 hr at 4°C with one fifth volume of packed sheep cells. Up to 10^8 human cells in 4 ml of R.P.M.I. (hepes) medium were added to 3ml of absorbed FCS and 5 ml of 10% (vol/vol) sheep erythrocytes. The E-rosettes were separated by centrifugation on 10 ml of Ficoll-Metrizoate as described in 3.2.4.1.

The non-rosetted cells were subjected to a second cycle of rosetting with neuraminidase-treated sheep erythrocytes to remove any remaining T-cells; thus giving a 'clean' non-T cell preparation. The rosetted cells from this step (En-rosettes) were discarded. Neuraminidase treatment is believed to enhance the stability of the E-rosettes without affecting the specificity (Webster & Asherson, 1974). A 2.5% suspension of sheep erythrocytes in PBS was incubated with 50 u/ml neuraminidase at 37°C for 20 min. These cells were washed three times and used for En-rosette formation on the same day.

3.2.4.3. Separation of sIg^+ cells by rosetting with Ox erythrocytes coated with anti-Ig

The procedure for isolating human sIg^+ cells was that described by de Gast & Platts-Mills (1979) which was developed from an earlier method for the isolation of rat sIg^+ lymphocytes (Parish & Hayward, 1974b). Goat anti-human antibodies (see 3.2.3.) were coated onto Ox erythrocytes using chromic chloride. A stock solution of 1% $CrCl_3$ in 0.85% NaCl was prepared, and the pH adjusted twice weekly to pH 5.0 with NaOH for three weeks. The use of 'aged' $CrCl_3$ gave more consistent results. The concentration of $CrCl_3$ required for coating the antibody to the Ox cells was predetermined for each

batch of cells. Freshly collected Ox red cells in Alsever's solution were washed five times in unbuffered 0.85% saline immediately before use. One hundred microlitres of diluted normal goat serum (adjusted to 0.1mg protein/ml) and 50µl of packed erythrocytes were added to 0.8ml of saline. Two-fold dilutions of $CrCl_3$ were made in 0.02 M sodium acetate buffer, pH 5.5, and 1.0ml volumes were added dropwise to the cell suspensions using a vortex mixer. After 10 min at 20°C, the reaction was stopped by the addition of 2.0ml of PBS. The coupled cells were washed twice and re-suspended in 2-3ml of PBS. The concentration of $CrCl_3$ immediately below that which caused microscopic agglutination was used as a guide to the concentration required when coating with the antibody solution (usually between 0.001 and 0.005%). Three concentrations around the predetermined concentration were then tested for coating the antibody solution (adjusted to 0.1mg protein/ml) to the Ox cells; and the highest concentration that did not cause micro-agglutination of the cells was used to prepare a larger batch of cells. Provided fresh Ox cells were used, and sterile precautions taken, the coated cells could be stored for up to 2 weeks in PBS at 4°C. Cell lysis or agglutination did not usually occur during this period of storage and the rosetting capacity of these cells was unimpaired.

'Non-T' cells were washed and resuspended in R.P.M.I. (hepes) with 10% FCS at a concentration of up to 20 x 10^6 cells/ml. An equal volume of 10% coated-Ox cells (vol/vol) was added to the cell suspension in an ice bath at 0°C. This mixture was spun at 200g for 7 min at 4°C. After making 0.1% with respect to sodium azide, the pellet was gently resuspended and allowed to warm to room temperature before separating the rosetted cells on a Ficoll-Metrizoate gradient containing 0.1% azide. Capping of the surface immunoglobulin was inhibited at 4°C; but once formed, the rosettes were stable at room temperature(Parish & Hayward,1974a). The low temperature

and the presence of azide also inhibited the formation of Fc-rosettes (Parish & Hayward, 1974a). The sIg^+ cell preparations contained less than 2% E-rosetting cells, but contained up to 20% monocytes as demonstrated by cytochemical staining for α-naphthyl esterase (deGast et al, 1979).

3.2.4.4. Determination of the 5'-nucleotidase activity of the mononuclear cell subpopulations.

Unless stated otherwise, all the cell preparations were cultured overnight before assaying for 5'-N activity. The cells were suspended in R.P.M.I. 1640 medium buffered with bicarbonate and supplemented with 2mM glutamine, 100 iu/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FCS (R.P.M.I. (bicarbonate)-FCS). After 16-18 hr incubation in plastic bijou bottles at 37°C in humidified air with 5% CO_2, the cells were washed three times in TBS and assayed for 5'-N in the usual manner.

3.2.5. Determination of the apparent Michaelis constant for 5'-Nucleotidase

The initial rate of reaction (Vi) for 5'-N on intact circulating mononuclear cells was determined for six concentrations of AMP ranging from 5-50 μM. The reaction mixture was the same as that for the routine 5'-N assay except that the concentration of β-glycerophosphate was raised to 150μM to ensure efficient inhibition of alkaline phosphatase activity at the higher substrate concentrations. The apparent Michaelis constant (Km) was calculated from plots of Vi against Vi/S, where S is the substrate concentration (Eadie, 1942). The Michaelis-Menten equation may be written in the form:

$$Vi = Vmax - Km.\frac{Vi}{S}$$

where Vmax = the maximum velocity of reaction. The Km is therefore given

as the slope of the plot of Vi against Vi/S. A computer program was used to determine the Km by the method of least squares, together with the standard error. The SEM was normally less than 10%.

3.2.6. Mixed cell cultures

Allogeniec cell-mixture experiments were performed by culturing equal numbers of circulating mononuclear cells from two individuals together in the R.P.M.I. (bicarbonate)-FCS culture medium described in 3.2.4.4. Cultures were set up in plastic bijou bottles with 2 x 10^6 cells in 1.5ml of medium. After incubating for 18 hrs at 37°C, the cells were washed three times in TBS and assayed for 5'-N.

3.2.7. Solubilization of cells with Triton X-100 detergent

Circulating mononuclear cells were washed and resuspended in TBS to give 10^5 - 10^6 cells in a volume of 0.45ml. The cells were lysed by the addition of 0.05ml of 10% (wt/vol) Triton X-100 detergent. The 5'-N activity was then determined in the usual manner in a final volume of 1.0ml.

3.2.8. Disruption of cells by freeze-thawing

Circulating mononuclear cells were washed and resuspended in TBS at a concentration of 2 x 10^6 cells/ml. Aliquots of 0.2ml were removed for 5'-N determination, and the remainder frozen to -20°C and stored overnight before thawing. Less than 5% of the cells remained intact after this treatment. 'Particulate' and 'soluble' fractions were obtained by centrifugation at 20 000g_{av} for 45 min at 4°C. The particulate fraction was then washed once in 10 mM tris-buffered H_2O, pH $7.5_{20°C}$.

3.3. RESULTS

3.3.1. A reassessment of the 5'-N activity of circulating mononuclear cells

Since the experiments in this section involved the measurement of the 5'-N activity of cells obtained from heparinized blood (rather than defibrinated blood), and because the cells were cultured overnight before assaying, the data on circulating mononuclear cell 5'-N have been re-presented in fig 13. The 5'-N activities shown here are appreciably higher than those obtained previously (see 2.4.2.1. , fig 9). However, comparison of the CV-H and X-H patients with the normal controls revealed the same pattern of results as obtained previously. Of the patients studied here, seven out of 14 CV-H patients and five out of eight X-H patients had a 5'-N activity below the normal range. In addition, the mononuclear cells from eight out of eleven cord blood samples had a 5'-N activity that was below the normal adult range.

3.3.2. The apparent Michaelis constant of 5'-nucleotidase with AMP as substrate

A typical Eadie plot, from which the apparent Km was determined, is shown in fig 14. The mean ($\pm$ s.d.) Km obtained with 12 healthy adults was 9.6 $\pm$ 2.7 μM AMP. As shown in fig 15, the Km values obtained with four X-H patients and six CV-H patients were all within the normal adult range; and the mean values were not significantly different from normal (Student's t-test, $p > 0.25$). Of the eight cord blood samples investigated, seven were within the normal range: the remaining sample gave a Km of 31.8 μM AMP (SEM = $\pm$ 3.9). The patient and cord blood samples investigated had a 5'-N activity that was at least one standard deviation below the normal mean.

Figure 13

5'-NUCLEOTIDASE OF CIRCULATING MONONUCLEAR CELLS

The vertical bar represents the 'normal range' of 5'-nucleotidase activity.

Figure 14

AN EADIE PLOT FOR THE DETERMINATION OF THE Km OF 5'-NUCLEOTIDASE

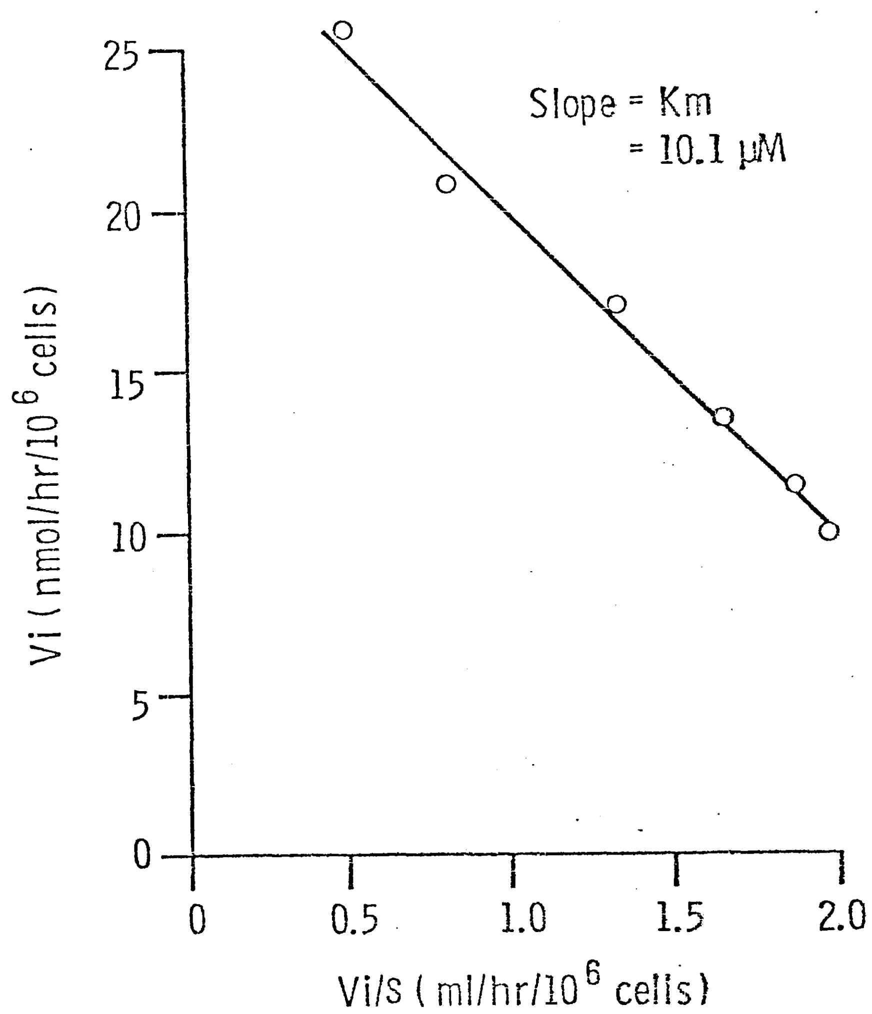
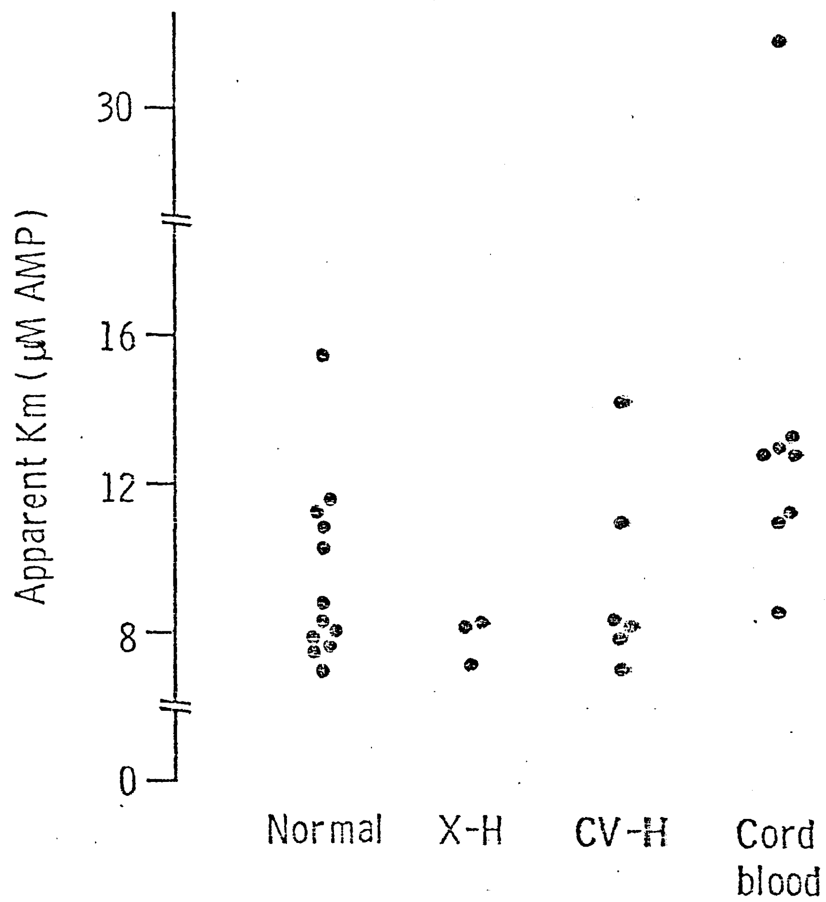


Figure 15

THE APPARENT Km OF 5'-NUCLEOTIDASE ON CIRCULATING MONONUCLEAR CELLS



The CLL patient, T.R., who had a supranormal level of 5'-N activity (fig 10, 2.4.2.1.) was found to have a normal Km of 11.6 μM AMP (SEM = ± 1.5).

3.3.3. Mixed-cell cultures : the effect on 5'-N activity

Table 4 summarizes the results of the experiments performed to investigate the possibility that circulating mononuclear cells may produce a soluble factor that regulates the 5'-N activity. When cells from CV-H patients or from cord blood were co-cultured for 18 hrs with cells from a healthy adult, the 5'-N activity of the cell mixture was always found to be close to the mean activity of the two cell populations when cultured alone. Similarly, co-cultures of cells from two healthy donors revealed that short-term cultures of allogeneic cell mixtures did not affect the 5-N activity.

3.3.4. The cellular location of 5'-Nucleotidase

Since the routine measurement of 5'-N activity is performed on intact cells, it is conceivable that low levels of 5'-N activity may be the result of some of the enzyme being located intracellularly. Disruption of circulating mononuclear cells with Triton X-100 detergent caused an increase in the 5'-N activity (table 5). However, neither cells from patients nor cells from cord blood showed a significant increase beyond that observed with cells from healthy adults. Circulating mononuclear cells from CV-H patients and healthy adults did not show an increase in 5'-N activity when disrupted by freeze-thaw (table 6). The 5'-N was located in the 'particulate' fraction of the cells from both CV-H patients and normal subjects.

3.3.5. The 5'-nucleotidase activity of normal mononuclear cell subpopulations

Mononuclear cells obtained from 20ml samples of normal adult blood were depleted of T-cells by two cycles of rosetting (3.2.4.2.). The 5'-N

TABLE 4.

5'-NUCLEOTIDASE ACTIVITIES (nmol AMP/hr/10^6 cells) OF CIRCULATING MONONUCLEAR CELL MIXTURES MAINTAINED IN CULTURE FOR 18 HRS.

	Experiment 1	Experiment 2
Control A alone	15.6	16.1
Control B alone	10.6	19.1
Control A + Control B	13.9 (13.1)	17.6 (17.6)
Patient alone	2.4	2.3
Patient + Control A	9.3 (9.0)	10.1 (9.2)
Patient + Control B	6.3 (6.5)	11.2 (10.7)

	Experiment 3	Experiment 4
Control C alone	14.7	13.6
Cord blood alone	4.7	3.8
Control + cord blood	7.9 (9.7)	8.1 (8.7)

Each experiment was conducted with different controls and patients. The figures in parentheses denote the expected mean 5'-N activities of the cell mixtures.

TABLE 5.

THE EFFECT OF TRITON X-100 DETERGENT ON THE 5'-NUCLEOTIDASE ACTIVITY OF CIRCULATING MONONUCLEAR CELLS

Source of cells	Mean $\frac{\text{Activity of solubilized cells}}{\text{Activity of intact cells}} \times 100 \pm$ s.d.
Normal	136 ± 8 (n=9)
Cord Blood	141 ± 9 (n=5) *
CV-H	147 ± 20(n=6) *
X-H	148 ± 23(n=4) *

* Not significant ($p > 0.1$ using Student's t-test).

TABLE 6.

5'-NUCLEOTIDASE ACTIVITY (nmol AMP/hr/10^6 cells) OF CELLS DISRUPTED BY FREEZE-THAWING

Subjects	Intact cells	Freeze-thawed cells		
		Homogenate	particulate* fraction	soluble fraction
NORMAL	17.9	17.5	16.6	0.4
	13.3	12.3	12.5	0.3
	12.9	11.5	10.7	0.3
CV-H patients	17.9	18.5	17.7	0.4
	9.4	7.4	6.7	0.1
	1.7	1.1	0.6	0.0

* The "particulate" fraction was obtained by centrifugation at 20 000g_{av} for 45 mins. and washing with 10mM tris-buffered H_2O, pH 7.5.

activity of the 'non-T' cells was not significantly different from the total mononuclear cell preparation (table 7). However, this type of experiment suffers from the drawback that the 'non-T' population consists of two major cell populations (B-cells and monocytes) that may have different 5'-N activities. Similar experiments were therefore performed with tonsil cell preparations which are nearly pure lymphocytes. Since tonsil lymphocytes initially show a significant, but inconsistent, decrease in 5'-N activity during culture (see 4.3.3.), it was decided to use tonsil lymphocytes that had been cultured for 2-4 days; by which time the 5'-N activity was relatively constant. These cells were separated into T- and 'non-T' populations by a single cycle of En-rosetting and assayed immediately for 5'-N activity. As shown in table 8, the T-cells had significantly less 5'-N activity than the unseparated cells, while the 'non-T' cells had significantly more 5'-N activity.

When both T- and B- cells were isolated from blood by positive selection (see fig 12; 3.2.4.), similar results to those with tonsil lymphocytes were obtained (table 9). The cells with neither B- nor T- cell markers (E^-sIg^- cells; presumably monocytes and 'null' cells) had a very low activity, although this was found to increase during culture (table 10).

3.3.6. The 5'-Nucleotidase activity of mononuclear cell subpopulations from cord blood and from patients with CV-H and X-H.

Because of the low numbers of B-cells present in many patients with CV-H, 100ml of blood was usually collected for the isolation of mononuclear cell subpopulations. Fifty millilitres was usually adequate for normal adults. With cord blood samples, sufficient numbers of cells were usually obtained from 25-30ml samples.

TABLE 7.

THE 5'-NUCLEOTIDASE ACTIVITY OF 'NON-T' CIRCULATING MONONUCLEAR CELLS.

Experiment	5'-N activity (nmol AMP/hr/10^6 cells)	
	Total circulating mononuclear cells	'Non-T' cells
1	13.9	15.2
2	14.3	13.0
3	18.0	21.4
4	18.7	15.9
5	25.7	25.7
6	26.5	31.5
7	27.7	32.5
8	28.0	25.8
mean $\pm$ s.d.	21.6 $\pm$ 6.0*	22.6 $\pm$ 7.5*

* Not significantly different (Student's paired t-test, $p > 0.25$).

TABLE 8.

THE 5'-NUCLEOTIDASE ACTIVITY OF TONSIL LYMPHOCYTE POPULATIONS

Experiment	5'-N activity (nmol AMP/hr/10^6 cells)		
	Unseparated cells	En^+ cells (T-cells)	En^- cells (B-cells)
1	7.2	4.3	29.7
2	10.8	7.1	22.9
3	13.1	5.9	45.8
4	13.7	9.4	44.9
5	14.4	3.7	22.4
6	14.9	11.1	25.5
7	15.9	6.3	30.8
8	19.7	6.3	54.0
mean ± s.d.	13.7 ± 3.7	6.8 ± 2.5*	34.5 ± 12.0*

* Values significantly different from the 5'-N activity of unseparated cells. (Student's paired t-test, $p < 0.001$).

TABLE 9

THE 5'-NUCLEOTIDASE ACTIVITY(nmol AMP / hr / 10^6 cells) OF CIRCULATING MONONUCLEAR CELL SUBPOPULATIONS ISOLATED FROM HEALTHY SUBJECTS

Total mononuclear cells	E^+ cells (T-cells)	E^-sIg^+ cells (B-cells)	E^-sIg^- cells
19.9 ± 5.9 (n=20)	13.5 ± 3.4 (n=15)	54.9 ± 13.8 (n=9)	4.3 ± 2.5 (n=8)

The values given are the mean ± s.d. for 'n' donors. E^-sIg^- cells were those remaining after the removal of E^+ and sIg^+ cells. All three subpopulations had a significantly different 5'-N activity to that of the total mononuclear cell preparation (Student's t-test, $p < 0.001$).

TABLE 10.

THE 5'-NUCLEOTIDASE ACTIVITY OF NORMAL PERIPHERAL BLOOD $E^{-}sIg^{-}$ MONONUCLEAR CELLS MAINTAINED IN CULTURE

Experiment	5'-N activity (nmol AMP / hr / 10^6 cells)		
	Day 0*	Day 1	Day 2
1	2.2	3.8	16.6
2	1.1	3.0	17.6
3	0.6	3.9	35.1

* The mononuclear cells were routinely measured on day one, but for these experiments the 5'-N assay was performed immediately after isolation of the cells (day 0), and after 18 and 42 hrs (days 1 & 2 respectively).

Isolation of E^+ and sIg^+ cells from CV-H patients revealed that the subnormal levels of 5'-N activity were not confined to a single lymphocyte subpopulation (table 11). Eight out of 13 CV-H patients showed a subnormal level of T-cell 5'-N activity, and four out of seven had a subnormal B-cell 5'-N activity. Four CV-H patients had insufficient numbers of B-cells for practical isolation. There was no consistent pattern of B- and T- cell 5'-N activity in the patients studied : three patients (A.W., S.C., & R.C.) had a low level of 5'-N activity on both B- and T- cells, while patient C.M. had a normal T-cell value and a low B-cell value, and patient G.R. had a low T-cell value and a normal B-cell value. The mean ($\pm$ s.d.) 5'-N activity of the E^-sIg^- cells from 12 CV-H patients was 5.6 ± 5.9 nmol AMP/hr/10^6 cells, which was not significantly different from the activity of these cells from normal blood (see table 9). Of the eight X-H patients studied, four had a low level of T-cell activity. No pattern of 5'-N activity was observed in the two families of X-H patients.

Cord blood B-cells consistently had less 5'-N activity on their B-cells than was present on the B-cells from healthy adults. However, only four out of seven samples had less 5'-N activity on the T-cells than adult T-cells; although they were all below the normal adult T-cell mean activity.

3.4. DISCUSSION

The apparent Km of around 10 μM AMP obtained here for human lymphocyte ecto-5'-N is in reasonable agreement with the values of 4-63 μM reported for purified 5'-N from various sources other than the cytoplasm of liver cells (Song & Bodansky, 1966; Widnell & Unkeless, 1968; Burger & Lowenstein, 1970; Sullivan & Alpers, 1971; Evans & Gurd, 1973; Nakamura 1976; Dornand, Bonnafous & Mani, 1978). There have been few reports of Km determinations on the enzyme from human sources. Song & Bodansky (1966)

Footnote to Table 11 :

Where too few (less than 2%) sIg^+ cells were present for isolation, this is indicated by (-), and n.d. denotes not determined.

* The CV-H patients, S.C., R.C., J.F. and C.M., were childhood-onset; the other nine CV-H patients were adult-onset.

** Denotes values within the normal adult range (mean ± 2 s.d.).

(a),(b). These X-H patients were brothers.

TABLE 11.

THE 5'-N ACTIVITY (nmol AMP/hr/10^6 cells) OF CIRCULATING MONONUCLEAR CELL SUBPOPULATIONS FROM PATIENTS AND CORD BLOOD

		Total mononuclear cells	T-cells (E^+)	B-cells (E^-sIg^+)
Normal adults (mean ± s.d.)		19.9 ± 5.9	13.5 ± 3.4	54.9 ± 13.8
CV-H patients*	E.O.	1.8	2.1	-
	B.H.	2.3	3.1	-
	A.W.	2.8	1.5	23.4
	D.B.	3.6	1.1	n.d.
	S.C.	5.1	5.7	8.9
	V.P.	5.6	2.7	n.d.
	G.R.	8.5**	3.9	78.0**
	R.C.	9.2**	5.4	24.9
	S.J.	10.1**	7.2**	34.6**
	C.F.	10.9**	7.9**	64.6**
	J.F.	18.1**	10.5**	-
	C.M.	21.6**	20.8**	17.4
	J.C.	22.3**	16.8**	-
X-H patients	M.R.	2.4	4.8	-
	G.B.(a)	3.8	4.1	-
	S.B.(a)	4.3	4.3	-
	A.W.(b)	7.4	3.6	-
	D.B.(a)	7.5	7.2**	-
	J.W.(b)	9.2**	9.9**	-
	C.L.	14.0**	13.6**	-
	D.C.	16.2**	12.4**	-
Cord blood	1	2.9	4.5	0.8
	2	7.2	5.9	10.1
	3	7.6	6.4	8.6
	4	7.7	6.1	9.3
	5	7.8	11.2**	15.9
	6	8.8**	9.0**	12.3
	7	13.3**	11.5**	13.2

obtained a value of 39 μM for 5'-N partially purified from liver, and Fox & Marchant (1976) reported a partially purified 5'-N from placentae to have a Km of 12-18 μM. In addition, since the completion of this kinetic study, Edwards et al (1978) have reported values of 20-29 μM for the Km of human lymphocyte 5'-N. Comparison of Km values in the literature should, however, be undertaken with caution, since certain buffers and detergents have been shown to affect the Km of 5'-N (Burger & Lowenstein, 1970; Nakamura, 1976). The Km appears to be independent of pH over the pH range 7.0 - 9.4 in the presence of Mg^{++}, and 7.0 - 8.7 in the absence of Mg^{++} (Levin & Bodansky, 1966).

In this study, no evidence has been found to suggest that the low 5'-N activity found in many CV-H and X-H patients was due to altered enzyme kinetics or to the production of a regulatory factor. This has since been confirmed on a limited scale by Edwards et al (1978) who found two patients with X-H to have a similar Km to two normals. Although one of the eight cord blood samples investigated here had a higher Km than the normal adult value, this obviously cannot be regarded as the general reason for cord blood mononuclear cells having less 5'-N activity than healthy adults.

Disruption of mononuclear cells discounted the possibility that the cells from patients have some of their 5'-N masked owing to an altered location within the cells. Since the cells from both patients and normals were observed to increase their 5'-N activity to the same extent on solubilization with detergent, the cause of the increased activity was not considered a major issue. However, in view of a recent report by Stanley, Edwards & Luzio (1979) this point warrants further discussion. The increase in 5'-N activity in the presence of detergent may occur for two reasons :

(a) The detergent may release latent enzyme activity from intracellular organelles such as vesicles.

(b) The detergent may stimulate the enzyme itself.

It has been suggested that approximately 20% of the 5'-N activity of rat liver cells is located in the membrane of vesicles within the cells, and that solubilization with detergent releases this activity (Stanley, Edwards & Luzio, 1979). However, this does not appear to be the case with human lymphocytes since the particulate fraction of freeze-thawed cells did not show an increase in 5'-N activity over the intact cells. This particulate fraction had been washed in hypotonic buffer to ensure that any vesicles present were permeable to AMP substrate. Nakamura (1976) showed that 5'-N purified from rat liver plasma membranes was stimulated by up to 50% by the detergent sodium deoxycholate, and that the Km was reduced three-fold. It therefore seems probable that the detergent was directly stimulating the enzyme of human mononuclear cells rather than exposing latent activity within the cell.

In common with the results of other workers (Quagliata et al, 1974; Kramers et al, 1976; Edwards et al, 1978), depletion of T-lymphocytes from blood mononuclear cells did not have a significant effect on the 5'-N activity. This has led to the belief that B- and T- cells have a similar 5'-N activity. However, similar depletion experiments with tonsil lymphocytes suggested that B-cells have a higher 5'-N activity than T-cells. These apparently contradictory results appear to stem from the differences in the constituent cell populations of the 'non-T' cell preparations. Typical figures for the composition of peripheral blood mononuclear cell are : T-cells 70%; B-cells 13%; and monocytes 16% (Gmelig-Meyling & Ballieux, 1977), although some authors believe the percentage of monocytes to be even higher (Zucker-Franklin, 1974; Rothbarth, Hendriks-Sturkenboom, & Ploem, 1976). However, single-cell suspensions of tonsil cells are nearly pure lymphocytes, of which about 40-60% are B-cells (Johnson, unpublished data; Janossy, de la Concha, Waxdal, & Platts-Mills, 1976). Thus, while

tonsil 'non-T' cells represent a relatively pure B-cell preparation, blood 'non-T' cells constitute at least 50% monocytes.

The positive selection of sIg^+ cells from peripheral blood 'non-T' cells showed that circulating B-cells have about four times more 5'-N activity per cell than circulating T-cells, and that the E^-sIg^- cells have a very low activity. The low activity on the monocytes was therefore 'diluting' the B-cell enzyme activity in the 'non-T' cell preparations. The increase in the 5'-N activity of E^-sIg^- cells during culture was consistent with the results obtained by Berman & Johnson (1978). These authors reported an 11-fold increase in the 5'-N activity of monocytes during four days of cultivation; with a peak activity on day 2. These observations may offer an explanation for the higher values of mononuclear cell 5'-N activity obtained for the normal donors in this chapter than the values given in chapter 2 (cf. fig 9, 2.4.2.1. and fig 13, 3.3.1.). The differences were not due to the method of cell collection (i.e. defibrinated or heparinized blood) since parallel samples collected by each method had the same 5'-N activity. The overnight culture of the cells studied in this chapter appeared to cause an increase in activity of unseparated mononuclear cells although the 5'-N activity of E^+ and sIg^+ cells remained unchanged.

The data presented in table 10 show that normal circulating mononuclear B-cells from adults have more 5'-N activity than T-cells. This observation is further supported by experiments conducted in co-operation with Dr.A.D.B. Webster at the Clinical Research Centre. Cytocentrifuge slide preparations of E^+ and sIg^+ cells were stained for 5'-N using the histochemical procedure described by Silber et al (1975). Three to 18 times more cells were found to stain positively for 5'-N in the B-cell preparations than in the T-cell preparations (table 12). Müller-Hermelink

TABLE 12.

HISTOCHEMICAL DETERMINATION OF 5'-NUCLEOTIDASE ON T- AND B-LYMPHOCYTES FROM NORMAL HUMAN BLOOD

Experiment	Percentage of positive cells	
	T cells	B cells
1(a)	3	22
2(a)	3	55
3(b)	22	53
4(b)	2	24
5(b)	8	21

(a) $sIgM^+$ lymphocytes were isolated

(b) sIg^+ lymphocytes were isolated

(1974) showed that the B-cell regions of human peripheral tissues sections could be distinguished from the T-cell regions by the histochemical demonstration of 5'-N activity. It therefore appears that the failure of previous workers to observe a difference between the 5'-N activity of B- and T- cells from human peripheral blood reflected the heterogeneity of the cell-composition of their B- and T- cell 'enriched' preparations. This is perhaps best illustrated by the work of Uusitalo & Karnovsky (1977b) on mouse spleen cells. These authors did not observe a significant difference between the 5'-N activity of B- and T- cell 'enriched' populations. However, when the spleen cells were labelled with ^{125}I-anti- Ig prior to staining for 5'-N histochemically, about 50% of the B-cells stained positively for 5'-N compared to only 10% of the T-cells. Barton & Goldschneider (1978) have recently reported that T-lymphocytes from the lymph nodes and spleen of rats have more 5'-N activity than the B-lymphocytes of these tissues. Although these experiments are open to criticism on the grounds that the purity of the 'enriched' populations was suspect, the histochemical staining of tissue sections showed the 5'-N activity to be associated with the T-cell dependent regions (c.f. Muller-Hermelink, 1974, for staining of human tissue sections).

Assuming mean B- and T- cell percentages of 13 and 70% respectively for normal human peripheral blood, B-lymphocytes account for about 40% of the total circulating mononuclear cell 5'-N activity, and the remaining 60% is due mainly to the T-lymphocytes. It is therefore apparent that the absence of circulating B-cells in patients with X-H and in some patients with CV-H has a significant effect on the mononuclear cell 5'-N activity. Similarly, the larger proportion of monocytes found in cord blood (Berman & Johnson, 1978) also results in a lower 5'-N activity of the mononuclear cells. In addition to the effects of cell-composition, reduced 5'-N activities on T- and B-lymphocytes were frequently observed. Patients with CV-H

were found to have a low 5'-N activity as a result of one or more of three mechanisms : low T-cell 5'-N activity, reduced numbers of B-cells, and low B-cell 5'-N activity. Patients with X-H often had a low T-cell activity in addition to their lack of circulating B-cells.

CHAPTER 4

THE SIGNIFICANCE OF ECTO-5'-NUCLEOTIDASE ACTIVITY ON FUNCTIONAL LYMPHOCYTES

4.1. INTRODUCTION

The experiments in this chapter were designed to investigate the consequences of low 5'-N activity in human lymphocytes. It is possible that the underlying cause of the immunodeficiency in some patients with hypogammaglobulinaemia is due to the failure of lymphocytes to migrate to lymphoid tissues such as the lymph nodes or spleen. Preliminary results using the 'capillary tube migration' technique to investigate the migration of cells in vitro (George & Vaughan, 1962) suggested that the circulating mononuclear cells from some CV-H patients showed poor migration (A.D.B. Webster, unpublished results). A more extensive investigation was carried out here, using the same technique, to study the migration of circulating mononuclear cells from both CV-H and CLL patients. The effect of inhibition of 5'-N activity on the migration of normal circulating mononuclear cells was also studied.

The phosphonate analogue of ADP, AOPCP, was used as an inhibitor of ecto-5'-N activity (Burger & Lowenstein, 1970, 1975). The stability of this analogue was investigated, since many of the experiments in this chapter involved adding AOPCP to cell cultures in order to inhibit the 5'-N for up to seven days. The effect of AOPCP on the in vitro transformation and immunoglobulin production in response to mitogens was investigated. In addition, changes in the ecto-5'-N activity of lymphocytes during transformation were studied in the absence of AOPCP. In this manner it was hoped to elucidate any possible involvement of the membrane enzyme in lymphocyte activation. Four different mitogens were employed in these transformation experiments: concanavalin A (Con A), phytohaemagglutinin (PHA), pokeweed mitigen (PWM) and protein A. PWM and protein A are known to stimulate populations of both B- and T- lymphocytes (Janossy et al, 1976; Ringdén & Rynnel-Dagöö, 1978). By analogy

with murine systems and from studies with T-cell deficient patients, Con A and PHA were originally considered to be specific activators of T-cells; but it is now clear that these mitogens also stimulate DNA synthesis in some B-cells (Phillips & Roitt, 1972 ; Chess, MacDermott, & Schlossman, 1974).

4.2. MATERIALS & METHODS

As in chapter 3, only the materials and methods not previously described are given here. Sterile precautions were taken where appropriate.

4.2.1. Materials

AOPCP (α β-methylene adenosine diphosphate)		SIGMA
Boric Acid		BDH
Bovine serum albumin		SIGMA
Mitogens: Con A	(source: Jackbean)	CALBIOCHEM CALIFORNIA
PHA	(source:Phaseolus spp.)	WELLCOME
Protein A	(source:Staph. aureus)	PHARMACIA
PWM		GIBCO
Mitomycin C	(source:Streptomyces Caspitosus)	SIGMA
Thymidine		BDH
6-methyl(^{3}H) thymidine		RADIOCHEMICAL CENTRE, AMERSHAM
Cristaseal		HAWKSLEY & SON LTD., SUSSEX
Culture flasks; Nunclon Delta Tissue Culture Flasks.		GIBCO
Microculture plates; Cooke Microtitre Flat-Bottom Tissue Culture plates).		STERILIN
Microcapillary tubes; 10 μl Microcaps		DRUMMOND SCIENTIFIC CO. USA.
Migration plates		STERILIN
Ultrafilter; PM 10, 10000 M.W. cut-off		AMICON

4.2.2. Assay for the concentration of AOPCP in cell cultures

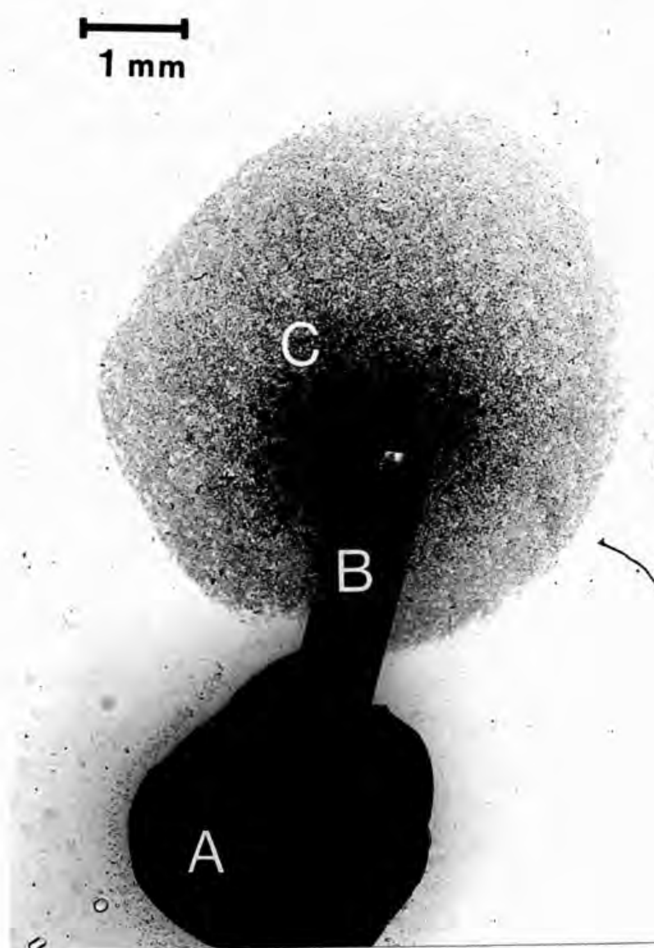
The ability of AOPCP to inhibit 5'-N activity was used to determine the concentration of the phosphonate in ultrafiltrates of spent culture medium. Five ml cultures containing 10^6 tonsil lymphocytes/ml in R.P.M.I. (bicarbonate)-FCS medium were set up in 10 ml culture flasks. These cultures, with or without 200 µM AOPCP added, were incubated in an upright position at 37°C in humidified air with 5% CO_2. After four or seven days, the cells were removed by centrifugation at 200g for 10 min. The supernatent was diluted with four volumes of TBS and ultrafiltrated through a 10000 M.W. cut-off membrane to remove enzyme molecules (particularly the 5'-N from the FCS and cell fragments). The concentration of AOPCP in the ultrafiltrates was determined by adding 0.3 ml aliquots to 0.2 ml of a tonsil lymphocyte suspension in TBS, and assaying for 5'-N activity. A standard curve for the inhibition of 5'-N by AOPCP was constructed for a range of concentrations up to 20 µM AOPCP. Control ultrafiltrates from cell cultures to which no AOPCP had been added were also tested for 5'-N inhibition.

4.2.3. The capillary migration technique

The method used to investigate the *in vitro* migration of circulating mononuclear cells was based on the procedure developed by George & Vaughan (1962) for guinea pig peritoneal exudate cells. Briefly, the cells were packed into a capillary tube and cultured for 18 hr, by which time the cells had migrated out of the tube and formed a fan-like monolayer on the culture plate (fig 16). The area of this monolayer was taken as an index of the cell migration.

Figure 16

MIGRATION OF CELLS OUT OF CAPILLARY TUBE CULTURES



Silicone grease (A) was used to fix the capillary tube (B) in position in the culture plate. Cells migrated out of the tube and formed a fan-like pattern (C).

Mononuclear cells were obtained by Ficoll-Triosil separation of 15 ml of fresh defibrinated blood. These cells were washed in R.P.M.I. (bicarbonate) culture medium with 10% human AB serum, and resuspended to a volume of 0.1 - 0.2 ml. This cell suspension was used to fill 10 µl 'microcap' capillary tubes which were then sealed at one end with 'Cristaseal'. The tubes were placed in a test-tube padded with cotton wool, and centrifuged at 200g for 7 min in order to pellet the cells in the capillary tubes. A diamond pencil was used to cut the tubes at the cell interface. The cut tubes were immediately placed in the individual wells of a migration plate, and fixed in position with silicone grease. The wells were filled with R.P.M.I. (bicarbonate) - AB serum medium, and sealed with silicone grease and microscope slide coverslips. After incubation of the migration plates for 18 hr at 37°C, they were placed in a Durst photographic enlarger and the image of the migration patterns projected onto paper for tracing. The traced areas were measured by planimetry, and the migration index (M.I.) determined as :

$$\text{M.I.} = \frac{\text{The mean area of migration of the test cells}}{\text{The mean area of migration of the control cells}}$$

4.2.4. Transformation of tonsil lymphocytes by mitogens : the effect on the lymphocyte 5'-nucleotidase activity

Tonsil lymphocytes in RPMI (bicarbonate)- AB serum medium were cultured at 37°C in Nunclon culture flasks at an initial cell density of 10^6 cells/cm^2. Ten or 50 ml capacity flasks were used in an upright position so that a cell concentration of about 10^6/ml was used. Individual 5'-N determinations were performed on separate cell cultures for each day; and not by withdrawing aliquots from the same cultures.

In the initial experiments involving dose-response curves and time-course observations for the four mitogens under investigation, 5ml cultures were set up and maintained at 37°C in humidified air with 5% CO_2. At the appropriate time, 0.2 ml aliquots were removed for the determination of the cell concentration using the Coulter Counter, and the remainder was washed 3 times in TBS before assaying for 5'-N activity. Cell aggregates were frequently found for the first three days in the cultures stimulated with either PHA or Con A. This problem was overcome by passaging the cell suspensions repeatedly through a syringe needle before washing in TBS, and filtering the final suspensions through nylon gauze. Whenever this treatment was necessary, the control cells were treated in the same manner.

B- and T- cells were isolated from 25ml cultures of tonsil lymphocytes after two or four days culture by a single rosetting step with neuraminidase-treated sheep erythrocytes. The cultured cells were spun on a Ficoll-Triosil gradient to remove any cells that may pass through the gradient (eg. dead cells). The interface cells were collected and washed once before adding to the En-rosetting mixture (see 3.2.4.2.). An aliquot of this cell suspension was removed, subjected to ammonium chloride lysis, and washed three times in TBS. This was the 'unseparated control'. The rest of the rosetting mixture was separated on a Ficoll-Metrizoate gradient to yield En^+ (T-cells) and En^- (B-cells). The cells were washed three times in TBS and assayed immediately for 5'-N.

4.2.5. Lymphocyte microcultures.

Microcultures of tonsil lymphocytes or peripheral blood mononuclear cells were set up for the studies on thymidine uptake (transformation) and immunoglobulin production. Cultures of 0.25 ml of cells

in R.P.M.I. (bicarbonate)-FCS medium (10^6 cells/ml), with or without mitogens, were set up in flat-bottomed microplates. The peripheral wells were not used for cultures; these were filled with water to prevent the evaporation that tends to occur in the outermost wells even in humidified incubators. The cultures were maintained at 37°C in humidified air with 5% CO_2.

4.2.5.1. Thymidine uptake

A stock solution containing one part (^{3}H)-thymidine (1.0 mCi/ml; 5 Ci/mmol) and one part 'cold' thymidine (5.0 mg/ml) was prepared and stored at -20°C. Eighteen hours before harvesting, 2.0 µl of the stock thymidine solution was added to the microcultures. The cells were harvested automatically using a Skatron (Norway) cell harvester. After two washes each with PBS, trichloroacetic acid, and methanol, the filters containing the acid-insoluble residues were thoroughly dried and placed in 5.0 ml of scintillation fluid (12.5 g PPO, 0.25 g POPOP, dissolved in 2.5 l toluene). The samples were left to dissolve for 2 days before counting for tritium.

4.2.5.2. Measurement of IgM in microculture supernates

The IgM present in the supernates of 7-day cultures stimulated with 10 µl/ml PWM was measured by the double antibody inhibition radioimmune assay (Platts-Mills & Ishizaka, 1975). The anti-sera and immunoglobulins were kindly provided by Dr.T.A.E. Platts-Mills (Dept. Immunological Medicine, C.R.C.) Rabbit antiserum against purified human IgM was made specific for µ-chains by repeated passages through human IgG

immunoabsorbent columns. Goat anti-rabbit IgG was passed over a human IgG immunoabsorbent column to remove cross-reacting antibodies. Human IgM was radiolabelled with ^{125}I according to the method of Klinman & Taylor (1969). A stock solution of borate-buffered saline (BBS) was prepared by making 360 g NaCl, 12.2 g Boric acid, and 19.5 ml of 1.0 M NaOH to 2 litres with distilled water. This stock solution was diluted 1:20 and adjusted to pH 8.0 with NaOH or HCl before use. A 3% solution (wt/vol) of bovine serum albumin in 100 ml of BBS was prepared (BBA), and used in the assay to reduce non-specific retention of immunoglobulins on the plastic walls of the assay tubes.

Appropriate dilutions (usually 1:6 and 1:66) of the culture supernates, and two-fold dilutions of standard IgM solutions (0.002 to 2.0µg/ml) were made in BBA, and 0.1ml aliquots used for assay. To these samples, 0.1ml of an optimum dilution of rabbit anti-human IgM and normal rabbit serum in BBA was added with shaking. With the reagents provided, a 1:1500 dilution of anti-Ig and a 1:60 dilution of normal rabbit serum was used. After leaving the mixtures for two hr at room temperature, 0.1ml of ^{125}I-labelled human IgM (2 ng IgM ; about 20,000 counts/min/ng) was added with shaking. After a further two hr at room temperature, 0.1 ml of a 1:4 dilution of absorbed goat anti-rabbit Ig antiserum was added. The samples were left overnight at 4°C, then washed three times with BBS at 4°C, and the radioactivity in the precipitate counted.

4.2.6. Inhibition of transformation by mitomycin C.

In some experiments, lymphocyte subpopulations were treated with mitomycin C in order to inhibit transformation (Bach & Voynow, 1966). The cells were incubated at 37°C for 30 min with 50 µg/ml mitomycin C in

in R.P.M.I. (hepes)-FCS medium, then washed three times in medium to remove the inhibitor before culturing.

4.2.7. Patients studied

The CV-H and CLL patients investigated here were described in chapter 2.

4.3. RESULTS

4.3.1. The inhibition of 5'-N activity by AOPCP : the stability of the inhibitor during cell culture

The presence of 50 μM AOPCP typically resulted in 80-90% inhibition of human lymphocyte ecto-5'-N activity (fig 17). This inhibition was reversible; full activity being restored by washing the cells in TBS. The inhibition of 5'-N was used to estimate the concentration of AOPCP remaining in the ultrafiltrates of spent cell cultures after 7 days incubation at 37°C. AOPCP was found to be stable under these conditions for at least 7 days (table 13).

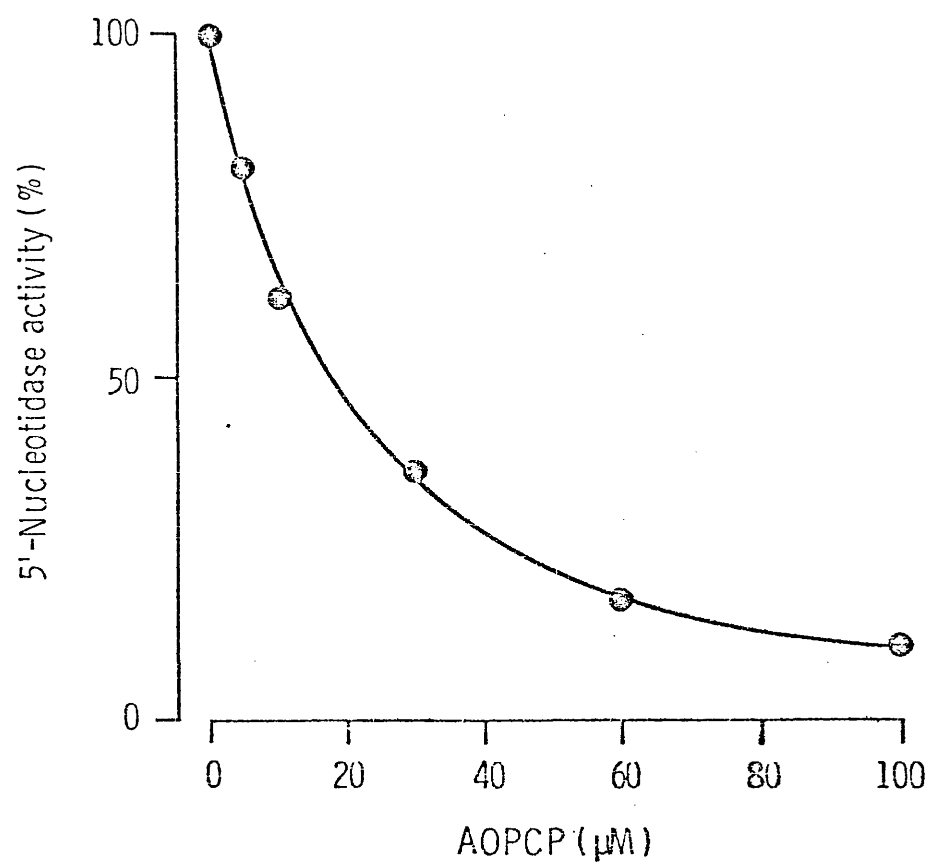
TABLE 13

THE STABILITY OF AOPCP DURING CELL CULTURE

Days in culture	Concentration (μM) of AOPCP (mean ± s.d. for 5 determinations)
0	198 ± 13
4	188 ± 16
7	212 ± 23

Figure 17

INHIBITION OF 5'-NUCLEOTIDASE BY AOPCP



4.3.2. The in vitro migration of circulating mononuclear cells

The migration of circulating mononuclear cells from patients and healthy individuals was investigated using the capillary tube culture technique. Because of the day to day variability of the results obtained with this procedure, the migration of cells from patients was always expressed as a fraction of the migration observed with control cells in the same experiment (ratio = M.I.). Two patients investigated on different days with the same control gave reproducible results when the migration was expressed as the M.I. When plotted on an appropriate logarithmic scale, the mean M.I. of 12 CV-H patients was not significantly different from unity, but the mean M.I. for nine CLL patients was significantly less than unity (fig 18). The CLL patient T.R., who had a supranormal level of 5'-N activity, had a typical M.I. of 0.48 for a CLL patient.

The presence of 250 μM AOPCP in the cell cultures did not affect the migration of normal circulating mononuclear cells (table 14). Lymphocytes isolated from normal circulating mononuclear cells migrated to a similar extent to the total mononuclear cell preparations (table 15).

TABLE 14

THE EFFECT OF AOPCP ON THE IN VITRO MIGRATION OF CIRCULATING MONONUCLEAR CELLS

	Relative area of migration ($\pm$ s.d. for n determinations)	
Control cells	100 $\pm$ 5 (n = 7)	
Cells + 250 μM AOPCP	97 $\pm$ 4 (n = 7)	$p > 0.05$

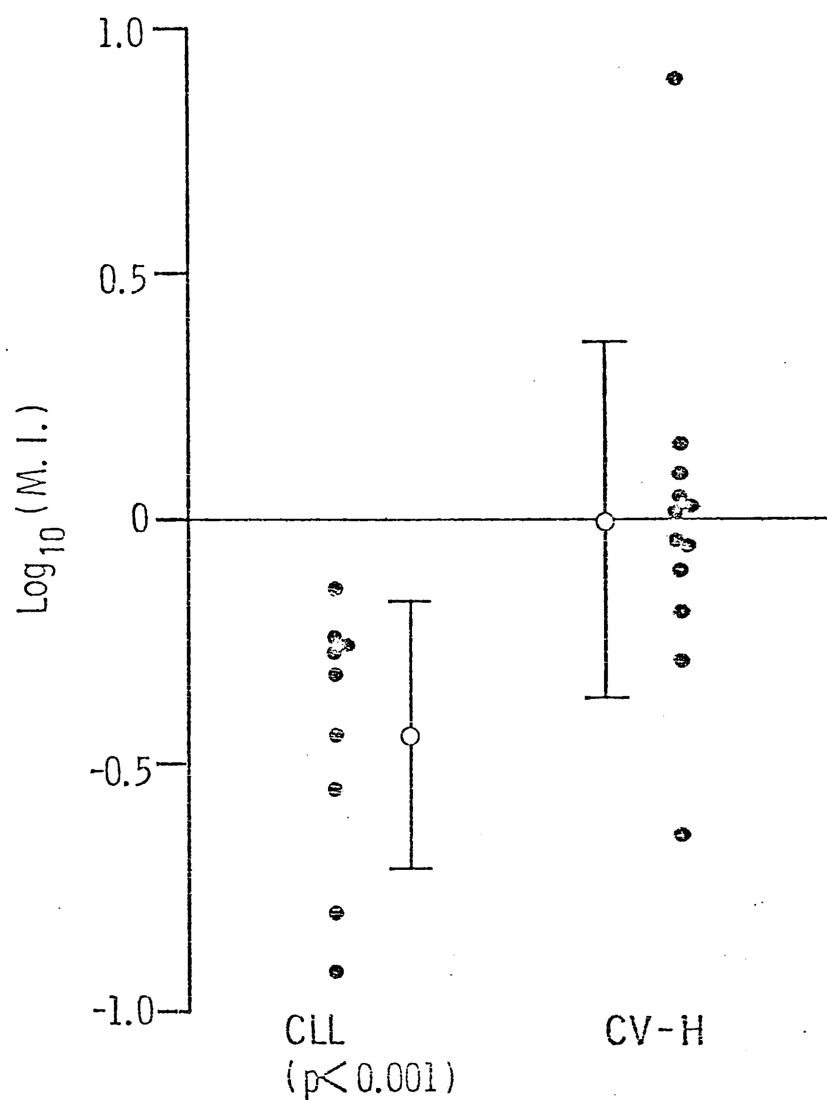
TABLE 15

THE IN VITRO MIGRATION OF CIRCULATING LYMPHOCYTES ISOLATED BY E-ROSETTING

	Relative area of migration ($\pm$ s.d. for n determinations)	
Total mononuclear cell population	100 $\pm$ 10 (n = 6)	
E-rosetting cells	93 $\pm$ 7 (n = 5)	$p > 0.05$

Figure 18

THE IN VITRO MIGRATION OF CIRCULATING MONONUCLEAR CELLS OBTAINED FROM PATIENTS WITH CLL AND FROM PATIENTS WITH CV-H



The vertical bars represent the mean ± s.d., and the p values denote the probability that the mean M.I. equals unity.

4.3.3. The effect of mitogenic transformation on human lymphocyte 5'-nucleotidase activity

Dose-response curves were constructed for the effect of each of the four mitogens (PWM, protein A, PHA, and Con A) on the 5'-N activity of tonsil lymphocytes by day 4 of culture (fig 19). The results were expressed as the ratio: $\frac{\text{5'-N per cell of stimulated cultures}}{\text{5'-N per cell of control cultures}}$ in order to facilitate the comparison of results obtained from different tonsil cultures. This was necessary because unstimulated tonsil cultures frequently, but inconsistently, showed a loss of 5'-N activity per cell during the first day of culture (table 16). The doses of PWM are given as a function of the volume of the dilution recommended by the suppliers. A suitable dose of each mitogen was used to construct time-course plots (fig 20). A decrease in 5'-N activity per cell was generally observed by the second or third day of culture in the presence of mitogen. This decrease in 5'-N activity just preceeded, or coincided with, the increase in cell numbers (fig 21). While protein A-, Con A-, and PHA- stimulated cultures continued to show a decrease in 5'-N activity till the fourth or fifth day of culture, PWM-stimulated cultures did not show any further reduction of 5'-N activity after the third day of culture.

TABLE 16

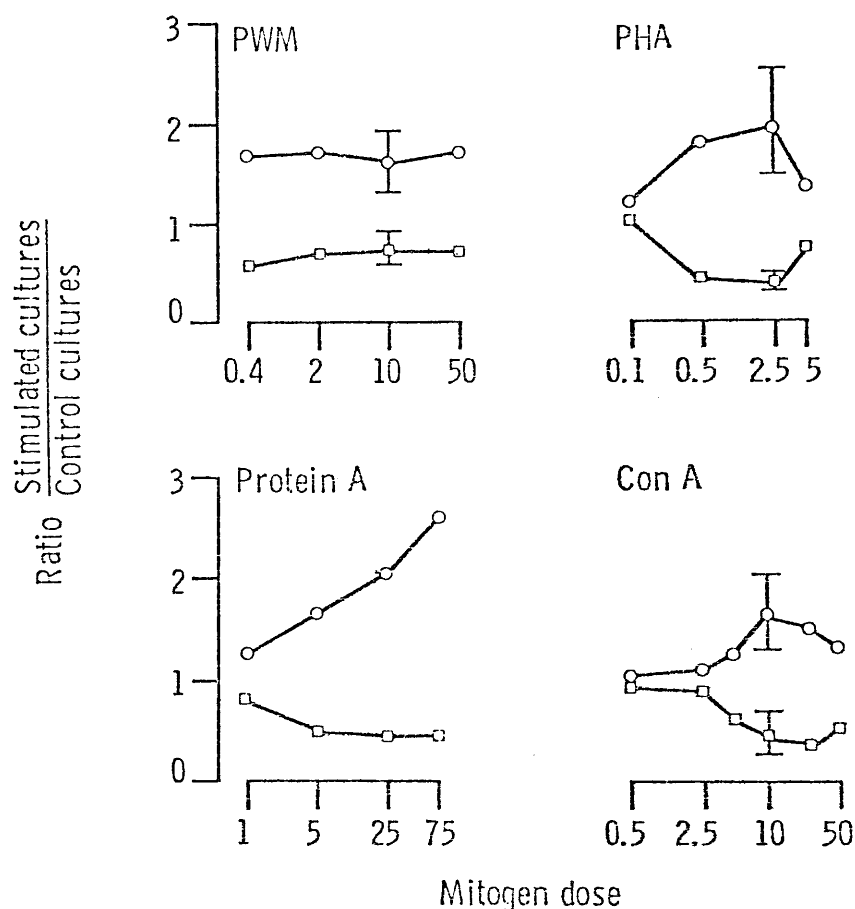
THE EFFECT OF CULTURE ON UNSTIMULATED TONSIL LYMPHOCYTES

Experiment	5'-N activity (nmol/hr/10^6 cells)				
	Day 0	Day 1	Day 2	Day 3	Day 4
1	43	31	33	39	39
2	34	15	n.d.*	14	n.d.
3	26	11	13	12	12
4	24	20	18	14	17
5	23	n.d.	27	32	24
6	22	12	15	17	15

*n.d. = not determined

Figure 19

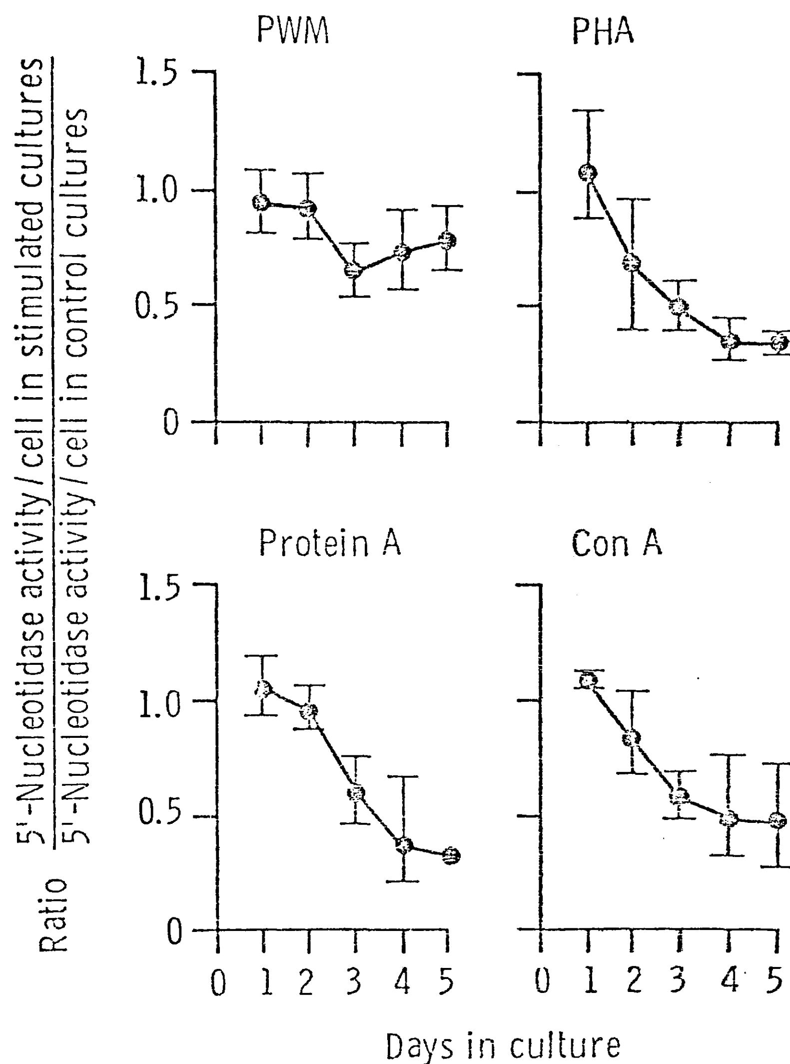
DOSE-RESPONSE PLOTS: THE EFFECT OF MITOGENS ON THE LYMPHOCYTE 5'-NUCLEOTIDASE AND CELL NUMBERS BY DAY 4 OF CULTURE



The effect of mitogenic stimulation on the cell concentration (O) and the 5'-nucleotidase activity per cell (□) by Day 4 of culture is shown. The mitogen dose is given as µg/ml for PHA, Protein A, and Con A ; and as µl/ml for PWM. The vertical bars represent the standard deviation of the geometric mean values from at least three experiments. The results shown for Protein A were obtained from a single experiment.

Figure 20

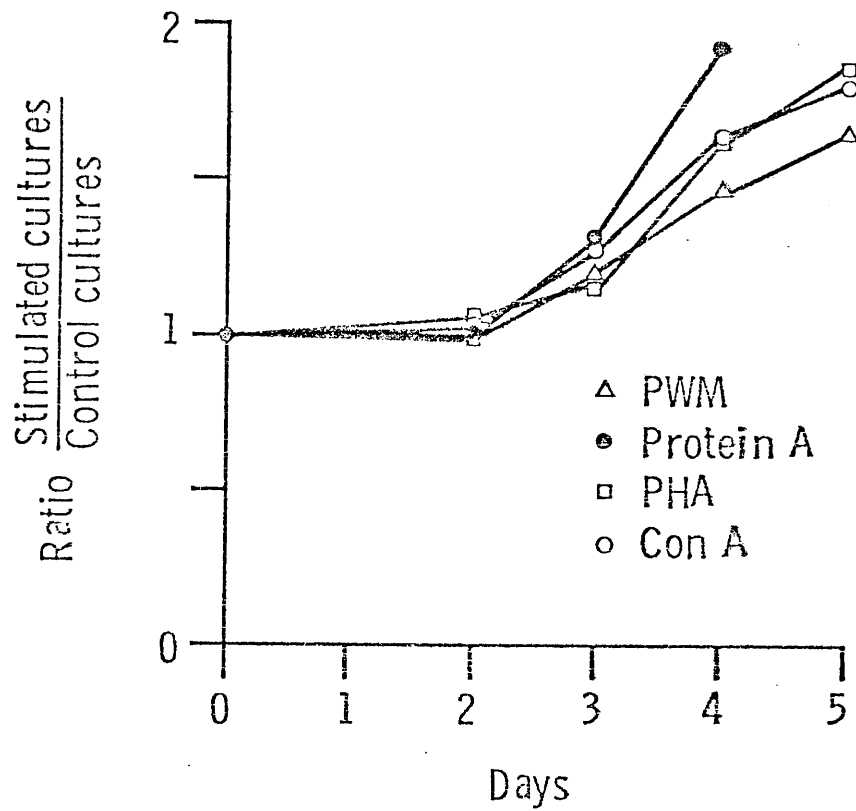
TIME-COURSE OF THE EFFECT OF MITOGEN STIMULATION ON THE 5'-NUCLEOTIDASE ACTIVITY OF TONSIL LYMPHOCYTES



The doses of mitogens employed were: PWM, 10 μl/ml ; PHA, 2.5 μg/ml ; Protein A, 25 μg/ml ; Con A, 10 μg/ml. The vertical bars represent the standard deviation of the geometric mean values from three or more experiments.

Figure 21

CELL PROLIFERATION IN MITOGEN-STIMULATED LYMPHOCYTE CULTURES



The geometric mean values of the ratio of cell concentrations from at least three experiments are given.

Both Con A and PHA are known to inhibit human lymphocyte 5'-N activity under certain conditions (Webster et al, 1978). However, the enzyme activity was unaffected, when tonsil lymphocytes were incubated with these mitogens (at the concentrations shown in fig 20) for one hour in R.P.M.I. (bicarbonate)-AB serum, before washing in TBS and assaying for 5'-N. The presence of serum in the incubation medium was responsible for relieving the inhibition by the mitogens; as shown by the results in table 17.

TABLE 17

THE INHIBITION OF LYMPHOCYTE ECTO-5'-NUCLEOTIDASE BY CON A

Conditions of experiment	Percentage inhibition of 5'-N activity by Con A	
	10 μg/ml Con A	100 μg/ml Con A
Cells incubated ± mitogen in:		
(a) R.P.M.I. (bicarbobate)	75	85
(b) R.P.M.I. (bicarbonate)+ 10% serum for 1 hr, then washed x 3 in TBS.	4	37
Cells assayed for 5'-N, ± mitogen in the assay.	73	80

Because the four mitogens employed are believed to have different specificities, it was decided to investigate the effect of each of these mitogens on the B- and T- cell 5'-N activity. Table 18 summarizes the results obtained with En^+ and En^- populations isolated by a single rosetting procedure (4.2.4.) from day 4 tonsil cultures. Perhaps surprisingly, Con A - transformed T-cells appeared to be unaffected, while the B-cells had considerably less 5'-N activity than B-cells from unstimulated cultures. All four mitogens caused a reduction of B-cell 5'-N activity; this reduction being more marked with the "T-cell mitogens",

TABLE 18

THE 5'-NUCLEOTIDASE ACTIVITY OF TONSIL T- AND B- CELLS ISOLATED FROM MITOGEN-STIMULATED CULTURES AFTER 4 DAYS

Mitogen	Total lymphocytes	T-cells	B-cells
PHA 2.5 µg/ml	3.9 ± 1.0 (13.3 ± 6.3)	3.0 ± 0.6 (5.5 ± 1.1)	7.4 ± 4.6 (43.2 ±12.4)
Con A 10 µg/ml	6.7 ± 3.0 (15.3 ± 6.3)	6.1 ± 2.8 (6.7 ± 0.6)	11.0 ± 1.5 (38.5 ±22.0)
PWM 10 µl/ml	10.4 ± 2.6 (13.3 ± 6.3)	7.4 ± 0.7 (5.5 ± 1.1)	17.1 ± 5.0 (43.2 ±12.4)
Protein A 25 µg/ml	5.5 ± 2.3 (14.7 ± 4.5)	2.4 ± 1.0 (7.6 ± 1.6)	18.4 ±10.2 (40.6 ±16.0)

The values shown are the 5'-N activities (nmol AMP / hr/ 10^6 cells) of cells recovered from 4-day cultures. The figures in parentheses are the activities of cells obtained from parallel unstimulated control cultures. The mean ± s.d. for three experiments is given.

PHA and Con A. PHA and Protein A both reduced the T-cell 5'-N activity, but PWM caused a slight increase. Time course plots of T- and B- cell 5'-N activity in cultures stimulated with PWM and Con A are shown in fig 22. With PWM, both T- and B-cell activities remained unchanged after two days of culture. However, Con A - stimulated cultures revealed a transient increase in T - cell 5'-N activity which preceeded cell-proliferation.

4.3.4. The effect of AOPCP on the ^{3}H-thymidine uptake of mitogen-stimulated cells

The uptake of ^{3}H-thymidine by mitogen-stimulated cells was used as a measure of cell transformation. Tonsil lymphocyte microcultures, with and without 200 μM AOPCP present, were fed ^{3}H-thymidine after 54 hr and harvested 18hr later. In order to avoid the possibility that the 'background' transformation of T-cells may mask the effect of AOPCP on B-cell transformation in PWM-stimulated cultures, T- and B- lymphocytes were isolated from tonsils by En-rosetting and unwanted transformation was inhibited by treatment with mitomycin C. Mitomycin-treated cells (i.e. Tm and Bm) were mixed with equal numbers of untreated cells, and stimulated with either 2.5 μg/ml PHA or 10 μl/ml PWM. The results in table 19 show that the transformation of both B- and T-cells was unaffected by the presence of AOPCP. The ^{3}H-uptake by PWM stimulated cultures was also investigated between 78-96 hrs, and the same results were obtained.

4.3.5. The effect of AOPCP on the immunoglobulin synthesis by PWM-stimulated lymphocytes

The effect of 250 μM AOPCP on the amount of IgM secreted by normal circulating lymphocytes was investigated. Since cell transformation is

Figure 22

EFFECT OF CON A AND PWM ON THE 5'-NUCLEOTIDASE OF TONSIL B- AND T- CELLS

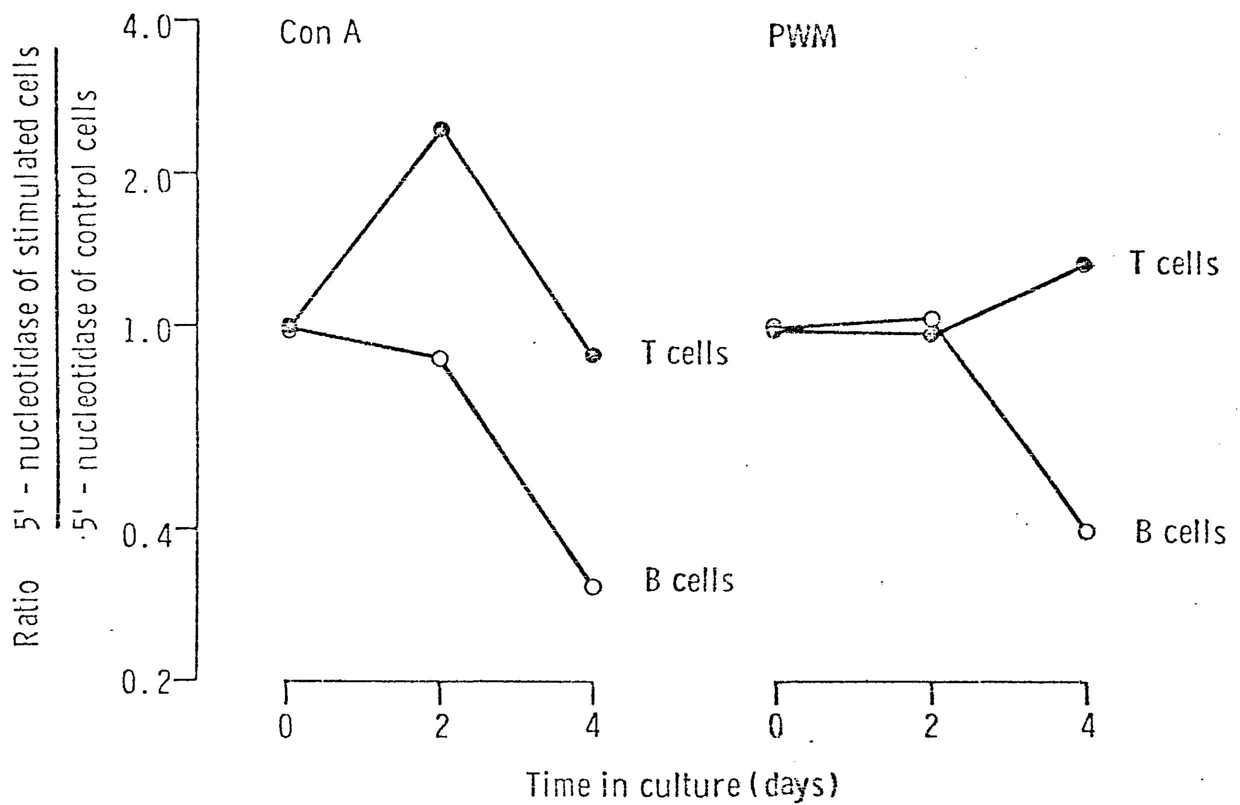


TABLE 19

THE EFFECT OF AOPCP ON THE ^{3}H-THYMIDINE UPTAKE BY MITOGEN-STIMULATED TONSIL LYMPHOCYTES

Cells in culture.	^{3}H-Thymidine uptake during 54 - 72 hrs of culture			
	PHA - stimulated cultures		PWM - stimulated cultures	
	- AOPCP	+ AOPCP	- AOPCP	+ AOPCP
T + B	8530 ± 2962 (19)	8804 ± 1792 (19)	7685 ± 1256 (17)	8388 ± 994 (19)
T + Bm	10415 ± 2390 (23)	10943 ± 2424 (24)	4532 ± 899 (10)	4326 ± 92 (10)
Tm + B	4726 ± 880 (10)	n.d.	3468 ± 152 (8)	3639 ± 172 (8)
Tm + Bm	216 ± 30 (0.5)	n.d.	160 ± 123 (0.4)	n.d.

The values given are the mean c.p.m. ± s.d. for quadruplicate determinations. Values in parentheses represent the stimulation index

$$= \frac{\text{c.p.m. of stimulated cultures}}{\text{c.p.m. of unstimulated cultures}}$$

n.d. = not determined.

a necessary prerequisite for in vitro immunoglobulin synthesis, the AOPCP was added to PWM-stimulated microcultures at day 0, 1, 2, or 4 in case transformation was affected in a manner that went undetected in the previous experiments (4.3.4.). The amount of IgM secreted into the triplicate supernates by day 7 was measured using the double antibody inhibition radioimmune assay. The results obtained with circulating mononuclear cells from three normal donors are summarized in table 20. The presence of 250 μM AOPCP, whether added on day 0, 1, 2 or day 4, did not affect the production of IgM in the microcultures.

TABLE 20

THE EFFECT OF AOPCP ON THE IgM PRODUCTION BY PERIPHERAL BLOOD LYMPHOCYTES STIMULATED WITH PWM

			IgM secreted (μg/ml)			
			AOPCP added to cultures at :			
Donor	PWM	AOPCP	Day 0	Day 1	Day 2	Day 4
	-	-	0.18			
B.P.	+	-	0.7			
	+	+	0.4	0.7	0.8	0.7
	-	-	0.06			
M.C.	+	-	0.9			
	+	+	1.2	0.9	1.1	1.1
	-	-	0.16			
M.J.	+	-	0.6			
	+	+	0.5	0.4	0.4	0.4

4.4. DISCUSSION

No evidence was found to support the idea that circulating mononuclear cells from CV-H patients migrate less well *in vitro* than those cells from healthy individuals. In addition, ecto-5'-N activity did not appear to be necessary for cell migration in this system. The poor migration observed with CLL cells may either reflect the composition of the mononuclear cell preparation, or a genuine impairment of migration that may be relevant *in vivo*.

Because of the negative result obtained with cells from CV-H patients, and because of the apparent non-involvement of ecto-5'-N in the migration of these cells *in vitro*, a thorough investigation of this technique was not undertaken. However, it should perhaps be mentioned that until the last decade, it was generally considered that lymphocytes did not migrate to a significant extent compared to macrophages. Thor et al (1968) reported that neither human lymph-node cells nor peripheral cells migrated freely using the capillary tube culture technique; although the lymph-node cells migrated well after 72 hr in culture. Sørberg & Bendixen (1967) had previously been successful in demonstrating the migration of human peripheral blood mononuclear cells using this technique. However, there still remained the possibility that the migrating cells were macrophage type cells (monocytes) rather than lymphocytes. Freidman et al (1969) reported that the cells from a number of murine tissues could be observed to migrate out of capillary tube cultures, and that lymphocytes and macrophages migrated to the same extent. The results in this chapter also show that human circulating lymphocytes (E-rosetting cells) migrate to the same extent as the total circulating mononuclear cell preparations.

In conclusion, it is not unreasonable to assume that lymphocyte migration was being observed in the experiments described in this chapter. However, the reason for the poor migration observed by CLL cells was not determined. It remains possible that, in this system, B-cells may not migrate to the same extent as T-cells. O'Neill & Parrott (1977) reported that human peripheral blood mononuclear cells show better random migration through filters in Boyden Chambers than do T-lymphocytes. However, in this system virtually no migration was observed by T-cells unless they had previously been cultured for 72 hr or had been activated by mitogens. Thus, direct comparison of results obtained with these two techniques cannot be made; and the relevance of these *in vitro* migration results to the situation *in vivo* is questionable.

A decrease in the 5'-N activity of human circulating mononuclear cells stimulated with PHA has previously been reported (Quagliata et al, 1974). These authors measured the 5'-N activity as a function of the total cell protein, and attributed the moderate decrease (25-50%) in the enzyme activity on day 5 of culture to the decreased surface membrane area per volume of the larger, stimulated, cells. This was shown not to be the case in the experiments performed here, since the 5'-N activity was measured as a function of the number of cells. The transient increase of T-cell 5'-N activity in Con A- stimulated cultures has an analogy in a murine system. Robinson (1978) and Johnson, Edwards & Robinson (1979) showed that Con A-stimulated spleen cell cultures had an increased 5'-N activity per cell on day 2, but by day 4 the activity was dramatically diminished.

Evaluation of the stability of AOPCP in cell cultures was of paramount importance since the inhibition of 5'-N by this compound is reversible by simply washing the cells. Its stability allowed its incorporation into

the cell culture medium in order to investigate the importance of 5'-N activity for transformation and immunoglobulin production by human lymphocytes. Selective inhibition of T- or B- cell transformation by treatment with Mitomycin C was necessary to determine whether either of these populations was selectively affected by AOPCP; especially in view of the apparent broad specificity of the mitogens. In conclusion, inhibition of 5'-N activity with AOPCP did not affect the transformation of B- or T- cells, and neither did it affect the production of immunoglobulin in PWM-stimulated cultures.

CHAPTER 5

GENERAL DISCUSSION

The discovery that ADA- and PNP- deficiency were the cause of the immune-dysfunction in some immunodeficient patients (see 1.6.2.) led to the speculation that the low levels of 5'-N activity may be the biochemical defect responsible for the immunodeficiency in some patients with hypogammaglobulinaemia. Normal adult B-lymphocytes have a high specific activity of 5'-N (3.3.5.), and since the circulating B-cells are either absent or functionally defective in X-H and CV-H patients, it seemed possible that the low 5'-N activity could be relevant to the aetiology of the disease. However, the results presented in section 3.3.6. show that the low 5'-N activities in these patients were not confined to the B-cell population. While the absence or reduced percentage of B-cells in X-H and CV-H patients contributed significantly to the low 5'-N activity of the total mononuclear cells, a low T-cell 5'-N activity was also observed in many patients. The number of CV-H patients with a low T-cell 5'-N activity was found to be in excess of the percentage of patients showing a functional defect of these cells (Webster & Asherson, 1974; de Gast et al, 1979) and no correlation was observed between the 5'-N activity and the ability of the patients' T-cells to provide 'help' for the *in vitro* production of immunoglobulin by normal B-cells (de Gast, Platts-Mills & Rowe, unpublished data). The B-cells from some CV-H patients had a normal level of 5'-N activity despite their functional defect. With the finding that inhibition of 5'-N activity did not affect the *in vitro* transformation or immunoglobulin synthesis of normal lymphocytes (4.3.4. and 4.3.5.), it now appears unlikely that the low level of 5'-N activity is directly responsible for the disease in CV-H and X-H.

At this stage it is not possible to rule out indirect effects of 5'-N activity on immune-function. For example, the recent report of the interaction of the enzyme with actin (Mannherz & Rohr, 1978) and the

presence of actin on the surface of lymphocytes, in particular B-lymphocytes (Owen et al, 1978), raise the possibility that the enzyme could be involved in the migration of lymphocytes in vivo. Although the results in this thesis do not provide any evidence for impaired migration of lymphocytes from CV-H patients, or any dependence of migration upon 5'-N activity (4.3.2.), it should be emphasized again that the results obtained with the in vitro technique may not adequately reflect the migration of cells in vivo.

Unlike ADA- and PNP- deficiency, in which the respective enzymes are completely absent or present in barely detectable quantities, the 5'-N deficiency in hypogammaglobulinaemic patients is only partial. With such a partial deficiency one might expect to find altered enzyme kinetics through a regulatory factor or a gene defect. However, no evidence was found to substantiate this view (3.3.2. and 3.3.3.). There appears to be fewer enzyme molecules present: as was found to be the case with CLL patients by using a fluorescent antiserum to 5'-N (La Mantia et al, 1977). It is still possible that there is an absence of subpopulations of both B- and T- lymphocytes that are particularly rich in 5'-N activity. If this is the case, future investigations with an appropriate specific antiserum to 5'-N may allow the separation of 5'-N-positive cells on the fluorescent activated cell sorter (Loken & Herzenberg, 1975) and the consequent characterization of these cells.

The change in 5'-N activity of tonsil lymphocytes during mitogen-induced transformation (4.3.3.) suggests that blast cells in vivo may have a different 5'-N activity to the circulating non-activated lymphocytes, and that the enzyme may be under some regulatory control. Of particular interest was the transient increase in the 5'-N activity of

Con A-stimulated lymphocytes before the onset of cell proliferation; thus possibly implicating the enzyme in the activation process. However, the inability of AOPCP to affect the mitogen activation of these cells contradicts this view. No explanation can be offered at this stage.

The effect of blast transformation on the level of 5'-N activity on tonsil lymphocytes was not unexpected in view of the number of reports demonstrating changes in the 5'-N activity during cell differentiation and development. Examples include: developing chick embryo brain (Manzoli, Ipata & Wegelin, 1971), developing rat sperm (Xuma & Turkington, 1972), regenerating rat liver (Masuda, Nishimura, Nojiri & Murano, 1976) and developing chick cartilage (Rodan, Bourret, & Cutler, 1977). In addition, the increase in monocyte 5'-N activity during in vitro cultivation (3.3.5.: Berman & Johnson, 1978) may reflect monocyte-macrophage differentiation. 'Activated' mouse peritoneal macrophages have less 5'-N activity than normal resident macrophages, and the level of the enzyme is very dependent upon the agent used to induce the inflammation (Edelson & Cohn, 1976).

There is circumstantial evidence which suggests that the low 5'-N activity found on the lymphocytes of many patients with hypogammaglobulinaemia may reflect a stage of maturation arrest in these cells. From functional, morphological and surface marker studies, the malignant cells of CLL patients are believed to be blocked at an early stage of differentiation (Salmon & Seligman, 1974; Catovsky et al, 1978). In this respect it is interesting that the CLL patient T.R., who had a supranormal level of 5'-N activity, had leukemic cells that appeared to be better differentiated than was characteristic of most CLL patients (see 2.4.1.4.). Both T- and B- lymphocytes from cord blood, which have less 5'-N activity than the

corresponding cells from adults (3.3.6.), are also considered to be immature (Hayward & Lawton, 1977). Further support for the 'maturation arrest' hypothesis may yet be gained from the isolation and study of bone-marrow B-cells which appear to be immature (de Gast & Platts-Mills, 1979).

The heterogeneity of 5'-N activity in CLL patients warrants some comment. It has been widely held that the clonal expansion of a 5'-N-negative or a 5'-N-positive cell gives rise to leukemic cells with a correspondingly low or high 5'-N activity (Silber et al, 1975; LaMantia et al, 1977). This view arose mainly from histochemical studies: the leukemic cells from most CLL patients do not stain for the enzyme, while those patients with positively-stained cells have a supra-normal level of 5'-N activity (Silber et al, 1975). It could be argued that those patients with high 5'-N activity possess cells that are blocked at a later stage of differentiation than the majority of CLL patients. This appears to be the case with the one patient studied in this thesis. This patient, T.R., has a 5'-N activity that is about 2-3 times the normal B-cell activity in healthy adults. The leukemic cells of this patient could be considered as having a 5'-N activity comparable with some normal B-cells since nearly 100% of the cells from this patient stained positively for 5'-N histochemically (Matamoros, unpublished data) compared with 22-55% of the B-cells from normal peripheral blood (table 12, 3.4.).

The high specific activity of 5'-N activity on normal circulating B-cells is probably relevant to the large variation of mononuclear cell 5'-N activity observed among normal individuals. The proportion of B-cells in normal circulating mononuclear cell populations has been reported to range from 4.3 - 26.2% in a sample of 25 subjects (Gmelig-Meyling & Ballieux, 1977). Using the 5'-N values shown in table 9 (3.3.5.) it can be calculated

that a mononuclear cell sample containing 20% B-cells will have about double the 5'-N activity of a sample containing only 5% B-cells.

The high 5'-N activity of B-cells has also been suggested to be the reason for the selective impairment of T-cell function in PNP-deficient patients and in a minority of ADA-deficient patients (Carson et al, 1979; Wortman, Mitchell, Edwards & Fox, 1979). These workers have investigated the effect of deoxy-adenosine on human B- and T-cell lines, and found the T-cells to be more susceptible to the toxicity of this deoxyribonucleoside. Of the cell lines investigated, 5'-N activity was higher in the B-cell lines than in the T-cell lines; other enzymes relevant to deoxy-adenosine metabolism were present in similar quantities in all cell lines. Although Webster et al (Manuscript in preparation, 1979) have since shown that this pattern of 5'-N activity is not true of all B- and T- cell lines, this does not affect the reasoning of these authors since the cell lines were merely being employed as models of high and low 5'-N activity in lymphocytes. 5'-N activity was suggested to be important in dephosphorylating the deoxy-AMP formed from added adenosine; thus preventing the accumulation of the toxic metabolite, deoxy-ATP. However, this proposed role for 5'-N depends on the enzyme being located within the cell. Carson et al (1979) showed that the enzyme of the cell lines studied was an ecto-enzyme, and the same is true of normal circulating lymphocytes. This appears to be a major drawback to this hypothesis. It is possible that a small percentage of the 5'-N activity is located within the cell and that this is functionally important. Alternatively, the active site of the enzyme may be accessible to both intracellular and extracellular substrate (Arch & Newsholme, 1978)

although there appears to be no evidence to support or contradict this view. Further investigations on the possible sites of action of the membrane-bound 5'-N will be necessary to elucidate this problem.

Arch and Newsholme (1978) have reviewed the evidence for a hormonal role of adenosine. These authors proposed that 5'-N, ADA and adenosine kinase were important in regulating the concentration of adenosine. Because this hypothesis necessitated an intracellular site of action of 5'-N activity, Arch & Newsholme raised the novel possibility that the membrane-bound enzyme may be capable of hydrolysing both intracellular and extracellular substrate. Drury (1936) suggested that adenosine may be involved in the control of blood flow, and it has since been shown that physiological concentrations of adenosine cause vasodilation or vasoconstriction in a number of mammalian organs (Berne, 1963; Scott, Daugherty, Dabney & Haddy, 1965; Haddy & Scott, 1968; Berne, Rubio & Curnish, 1974). Seegmiller, Watanabe & Schreier (1977) have reviewed the effect of adenosine on *in vitro* lymphocyte function. Exogenous adenosine enhances cell proliferation and antibody production at low concentrations, but inhibits at concentrations of around 1 mM or more. The presence of an inhibitor of ADA lowers the concentration at which adenosine becomes inhibitory. Of particular interest here is the observation that PHA-stimulation of human lymphocytes resulted in an increase in the rates of phosphorylation and deamination of adenosine, together with an increase in the amount of ADA secreted into the extracellular medium. This, together with the reduction of 5'-N activity of PHA-stimulated lymphocytes observed in this thesis (4.3.3.) suggests a concerted mechanism to reduce the concentration of adenosine during transformation.

At high concentrations of adenosine, the relative rates of phosphorylation and dephosphorylation suggest that the ADA is acting as a spillway for removing inordinately high concentrations of adenosine.

Burnstock (1972, 1977) has postulated the presence of 'purinergic nerves' supplying a number of organs, including the gastrointestinal tract, lung, trachea, seminal vesicles, bladder, oesophagus, eye, and probably parts of the cardiovascular and central nervous systems. ATP was shown to be the most likely neurotransmitter, and a role for 5'-N was proposed in the inactivation process. The enzymes, ATP-ase, 5'-N, and ADA are together capable of rapidly breaking down ATP to pharmacologically inactive compounds. In this case, the proposed role for 5'-N is consistent with the extracellular site of enzyme action. However, it remains to be seen whether a similar role exists for the lymphocyte enzyme in, for example, intracellular communication.

In conclusion, no evidence was found to implicate the low levels of 5'-N activity as the cause of the immune-dysfunction in CV-H and X-H patients. Rather, the 5'-N activity appears to reflect the absence of certain cells and the 'immaturity' of some of those cells present. The significance of these results as regards the function of the enzyme is not immediately apparent.

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