STUDIES ON THE METABOLIC NATURE OF INDUCED CELLULAR DIFFERENTIATION OF LEUKAEMIC CELLS USING HL60 CELLS AS A MODEL

By

Nuzhat Ahmed MSc (Leuven)

A thesis submitted for the degree of **Doctor of Philosophy** in the Division of Biochemistry and Molecular Biology, School of Life Sciences at The Australian National University. February, 1993.

Dedicated to the loving memory of my father

Declaration

This thesis reports the results of research carried out in the Division of Biochemistry and Molecular Biology, School of Life Sciences, Faculties at The Australian National University, Canberra, under the guidance of Dr M.J. Weidemann. The materials incorporated in this thesis is my own work and has not been submitted towards a degree in any other Institution or University.

Nuzhat Ahmed

Table of Contents

Acknowledgements

I would like to express my deep gratitude to my supervisor Dr M.J.Weidemann for his useful, valuable and inspiring guidance at all steps of my PhD research. His interest and encouragement has made this project a memorable experience.

I also wish to thank Dr M.J.Howell for undertaking the enormous task of reading my thesis and offering useful suggestions.

Thanks to Lesley Maxwell, Electron Microscopy Unit, John Curtin School of Medical Research, for helping me with the electron microscopy experiments. Special thanks go to Noel Call for helping me with the preparation of the photographic material. I would like to express my appreciation to all members of the Division of Biochemistry and Molecular Biology, School of Life Sciences, in particular Bill Nicholson and Alan Crawford, for their various contributions and able assistance when needed.

I am grateful to the Australian National University for offering me a postgraduate scholarship and to the University of Dhaka, Bangladesh, for granting study leave that enabled me to complete this thesis.

Many thanks are due to my parents, brothers and sister, whom I have missed greatly during this period. Their support and encouragement brought me through some difficult times. My special appreciation and gratitude go to my husband Kamran for his constant support, cooperation and encouragement. Navera, our two year old daughter, had to forebear my lack of constant attention, but not affection. Special thanks to her.

List of publications

(1) "Oral Cholera vaccines containing B-subunit-killed whole cells and whole cells only. Cross reacting antigens of the members of family *Vibrionaceae* and the vaccines" I. Ciznar, N. Hussain, C. R. Ahsan, B. A. Kay, J. D. Clemens and D. A. Sack. in Vaccine 1989, **7**, 111-116.

(2) "Differentiation in the promyelocytic HL60 cell line: Effect of dimethylsulfoxide, butyrate and phorbol ester" Nuzhat Hussain, John. F. Williams and M. J. Weidemann. in Proc. Aust. Biochem. Soc. 1990, **22**, SP64.

(3) "The Human promyelocytic HL60 cell line: A model of myeloid cell differentiation using dimethylsulphoxide, phorbol ester and butyrate". Nuzhat Ahmed, John. F. Williams and M. J. Weidemann. in Biochemistry International, 1991, **23/3**, 591-602.

(4) "Glycolytic, glutaminolytic and pentose-phosphate pathways in promyelocytic HL60 and DMSO-differentiated HL60 cells". Nuzhat Ahmed, John. F. Williams and Maurice. J. Weidemann. in Biochemistry and Molecular Biology International, 1993, **29**, 1055-1067.

(5) "Purine metabolism in promyelocytic HL60 and dimethylsulphoxide-differentiated HL60 cells". Nuzhat Ahmed and Maurice. J. Weidemann. In press.

(6) Biochemical effect of three different inhibitors of purine/pyrimidine metabolism on differentiation in HL60 cells. Nuzhat Ahmed and Maurice. J. Weidemann. Submitted for publication in Leukaemia Research.

(7) "Production of reactive nitrogen intermediates by HL60 and dimethylsulphoxide-differentiated HL60 cells". Nuzhat Ahmed and Maurice. J. Weidemann. Submitted for publication in Leukaemia Research.

Abstract

Prolonged treatment of promyelocytic HL60 cells with dimethylsulphoxide, butyrate and phorbol 12-myristate 13-acetate (PMA) produced cells phenotypically similar to human neutrophils, monocytes and macrophages. The extent of differentiation was measured by the release of reactive oxygen species that accompany the oxidative burst upon stimulation of the cells with appropriate stimuli. Using PMA as a stimulus, the rates of superoxide production and luminol-dependent chemiluminescence (CL) in the presence of horseradish peroxidase by dimethylsulphoxide and butyrate-differentiated cells were not significantly different from those observed in neutrophils and monocytes isolated from normal peripheral human blood. However, in the absence of horseradish peroxidase, the luminol-dependent chemiluminescence in dimethylsulphoxide and butyrate-differentiated cells was significantly lower than that of the control blood cells, reflecting impaired expression of myeloperoxidase in the differentiated cells. In contrast, HL60 cells differentiated with PMA failed to show significant enhancement of superoxide production or luminol-dependent chemiluminescence even though the cells resembled macrophages morphologically. Impaired release of lysosomal enzymes was observed in all of the chemically-differentiated cells, suggesting deficiences in the differentiation process that may result in cells with defective azurophilic degranulation.

Measurement of reactive nitrogen intermediates, performed following stimulation of the cells with PMA in the presence of L-arginine, showed an increase of approximately 40% in the production of nitrate in DMSO-differentiated cells compared to 6% in HL60 cells. The rate in the DMSO-differentiated cells was the same as that in neutrophils separated from human blood. L-N^G monomethyl arginine (L-NMMA), an inhibitor of nitric oxide synthesis by nitric oxide synthase, inhibited the production of nitrate in DMSO-treated cells and human PMNs. Interaction of reactive nitrogen intermediates with reactive oxygen intermediates was inferred from the effect of added L-arginine on luminol-dependent chemiluminescence and

cytochrome C reduction: PMA-stimulated HL60 cells showed enhanced CL and a decreased rate of cytochrome C reduction in the presence of increasing concentrations of L-arginine, both of which were reversed by L-NMMA in a concentration-dependent manner. In contrast, DMSO-differentiated cells showed decreased rates of CL and cytochrome C reduction with increasing concentrations of L-arginine, which were relieved to some extent by L-NMMA in a dose-dependent manner. Human PMNs behaved like DMSO-treated cells with respect to the formation of reactive nitrogen intermediates. A possible role for the peroxynitrite anion ($ONOO^-$) has been suggested to explain the differential enhancement and inhibition of the CL response observed in the case of undifferentiated HL60 cells, DMSO-treated cells and human PMNs.

Both HL60 and chemically-differentiated cells gave rise to tumours when 4×10^7 cells were transplanted into nude mice, suggesting either defective differentiation or the presence of a substantial population of differentiation-resistant cells in the HL60 cell line. The pattern of tumour growth in response to the injection of HL60 cells and chemically-differentiated cells was different when the site of transplantation was changed. Subcutaneous transplantation produced massive tumours confined to the site of injection, while intraperitoneal injection of cells was followed by the development of ascites and the appearance of small solid tumours in the peritoneal cavity just above the bladder. When cell numbers as low as 3×10^6 were injected, the incidence of tumour growth decreased significantly and the survival times of tumour-bearing mice were increased when differentiated cells were injected compared to those recorded with undifferentiated HL60 cells. Phenotypic characteristics of tumour cells were significantly different to those of the cells from which they were derived.

There was no significant difference in the rate of utilization of exogenous glucose between HL60 and DMSO-differentiated cells. The activities of the glycolytic enzymes were similarly unaffected. In contrast, the activity of the oxidative segment of the pentose-phosphate pathway was enhanced by differentiation, as evidenced by increased activities of glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and increased oxidation of

[1-^{14}C]-glucose compared that of [6-^{14}C]-glucose in DMSO-treated cells. When exogenous glutamine was present as a cosubstrate, glucose utilization was depressed but, at the same time, glutamine utilization was enhanced by glucose in both cell types. The decreased utilization of glucose in the presence of glutamine is consistent with allosteric inhibition of phosphofructokinase, the rate-limiting step in glycolysis. Even though HL60 cells had twice the total glutaminase activity of their differentiated counterparts, the uptake of glutamine by these cells was low, especially when it was the sole substrate. The stimulation of glutaminolysis by glucose may be due to activation of mitochondrial glutamine transport. A large proportion of the glutamine utilized by both HL60 and DMSO-treated cells could be accounted for in the net accumulation of glutamate, aspartate and alanine, while up to 35% was oxidized to CO_2. In contrast, a very high proportion of the glucose utilized was converted to lactate and very little was oxidized. Hence, glycolysis and glutaminolysis in HL60 and DMSO-differentiated cells may not only contribute to the production of energy but may provide, in the undifferentiated HL60 cells, substrates for biosynthetic processes that utilize the intermediates of these pathways as precursors (e.g., purine and pyrimidine synthesis) or, in the case of differentiated cells, substrates that maintain NADPH in a reduced state for the synthesis of reactive oxygen intermediates.

Studies on purine and pyrimidine metabolism in HL60 and DMSO-differentiated cells showed that HL60 cells produced purines *de novo* at six-fold the rate of the differentiated cells and at three times the rate measured in PMNs separated from human blood. This is consistent with significantly higher activities of the key regulatory enzymes of the *de novo* purine synthesis pathway in HL60 cells. In a similar way the rate of *de novo* pyrimidine synthesis fell by approximately three-fold following differentiation by DMSO. There was an active purine salvage pathway in HL60 cells which remained unaltered by DMSO-differentiation. The activities of the salvage enzymes were also the same after differentiation. Human PMNs had an active purine salvage pathway, but its rate was five times lower than that of HL60 cells, consistent with much lower activities of the salvage pathway enzymes.

Since differentiation of HL60 cells with DMSO resulted in decreased ability to synthesize purine nucleotides *de novo*, the effect of four different nucleotide biosynthesis inhibitors on differentiation and purine/pyrimidine metabolism was studied in HL60 cells. Among the inhibitors used, acivicin (an antimetabolite and a glutamine analogue) and mycophenolic acid, (a specific inhibitor of IMP dehydrogenase and GMP synthetase) were able to differentiate HL60 cells (as evidenced by increased production of reactive oxygen species) while alanosine (a specific inhibitor of adenylosuccinate synthetase) and dipyridamole (an inhibitor of nucleoside transport) failed to do so. The induction of differentiation in HL60 cells by acivicin and mycophenolic acid was associated with a substantial decrease in both the guanylate and adenylate pools and appeared to be dependent on the depletion of the intracellular GTP pool. Even though treatment of HL60 cells with alanosine and dipyridamole also resulted in guanylate depletion, this effect was secondary to the depletion of adenylates and occurred only after prolonged exposure of the cells to inhibitors. Therefore, it is proposed that the depletion of guanine ribonucleotides resulting from the inhibition of either *de novo* purine biosynthesis (in the case of acivicin treatment) or following the specific inhibition of *de novo* guanine nucleotide biosynthesis alone (in the case of mycophenolic acid treatment) may play a role in initiating the complex mechanism of differentiation in HL60 cells.

List of figures:

List of tables:

List of enzymes:

Recommended name of the enzymes	Enzyme commission number
Acid Phosphatase	3.1.3.2
Aldolase	4.1.2.7
Amidophosphophoribosyl transferase	2.4.2.14
Adenine phosphoribosyl transferase	2.4.2.7
Adenosine deaminase	3.5.4.4
Adenylate kinase	2.7.4.3
Adenylosuccinate synthetase	6.3.4.4
Alanine amino transferase	2.6.1.2
Aspartate amino transferase	2.6.1.1
Carbamoyl phosphate synthetase	6.3.5.5
Citrate synthase	4.1.3.7
CTP synthetase	6.3.4.2
DNA polymerase	2.7.7.7
Fructose 1,6 diphosphatase	3.1.3.11
Glucokinase	2.7.1.2
Glucose 6-phosphate dehydrogenase	1.1.1.49
Glucose 6 phosphatase	3.1.3.9
Glutaminase	3.5.1.2
GMP synthetase	6.3.5.2
Guanylate cyclase	4.6.1.2
Hexokinase	2.7.1.1
Horse-radish peroxidase (HRP)	1.11.1.7
Hypoxanthine guanine phosphoribosyl transferase	2.4.2.8
IMP dehydrogenase	1.1.1.205
Lysozyme	3.2.1.17

Myeloperoxidase	1.11.1.7
NADP-linked malic enzyme	1.1.1.40
Nitric oxide synthase	1.14.23
5' Nucleotidase	3.1.3.5
Ornithine carbamoyltransferase	2.1.3.3
Phosphofructokinase	2.7.1.11
Phosphogluconate dehydrogenase	1.1.1.44
Protein kinase C	2.7.1.37
PRPP synthetase	2.7.6.1
Purine nucleoside phosphorylase	2.4.2.1
Pyruvate dehydrogenase	1.2.41
Pyruvate kinase	2.7.1.40
Ribonucleotide-diphosphate reductase	1.17.4.1
Ribonucleotide-triphosphate reductase	1.17.4.2
Superoxide dismutase	1.15.1.1
Transaldolase	2.2.1.2
Transketolase	2.2.1.1
Thymidine kinase	2.7.1.21
Tyrosine specific-protein kinase	2.7.1.112
Thymidylate synthetase	2.1.1.45
Xanthine oxidase	1.1.3.22

List of abbreviations

Abbreviations used	Recommended names
APRT	Adenine phosphoribosyl transferase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
cAMP	3', 5'-cyclic adenylic acid
cGMP	3', 5'-cyclic guanylic acid
CL	Chemiluminescence
CSF	Colony stimulating factor
CTP	Cytidine triphosphate
DMSO	Dimethylsulphoxide
DAG	Diacylglycerol
EPO	Erythropoietin
G-CSF	Granulocyte colony stimulating factor
GDP	Guanosine diphosphate
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
HBSS	Hank's balanced salt solution
HGPRT	Hypoxanthine guanine phosphoribosyl transferase
HRP	Horse-radish peroxidase
IL-1	Interleukin-1
IL-3	Interleukin-3
IL-5	Interleukin-5
IL-6	Interleukin-6
IP_3	Inositol 1,4,5 triphosphate
L-NMMA	L-N^G monomethyl arginine

MBP	Major basic protein
M-CSF	Macrophage colony stimulating factor
MPO	Myeloperoxidase
NADP	Nicotinamide adenine dinucleotide
PAS	Periodic acid schiff's staining
PBS	Phosphate-buffered saline
PI	Phosphatidyl inositol
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PMA	Phorbol 12-Myristate 13-Acetate
PMNs	Polymorphonuclear leukocytes
PKC	Protein kinase C
PRPP	1, Pyrophosphorylribosyl 5-phosphate
RA	Retinoic acid
Rib 5-P	Ribose 5-phosphate
ROI	Reactive oxygen intermediates
RTK	Tyrosine-specific protein kinase
SOD	Superoxide dismutase
TNF	Tumour necrosis factor
UTP	Uridine triphosphate

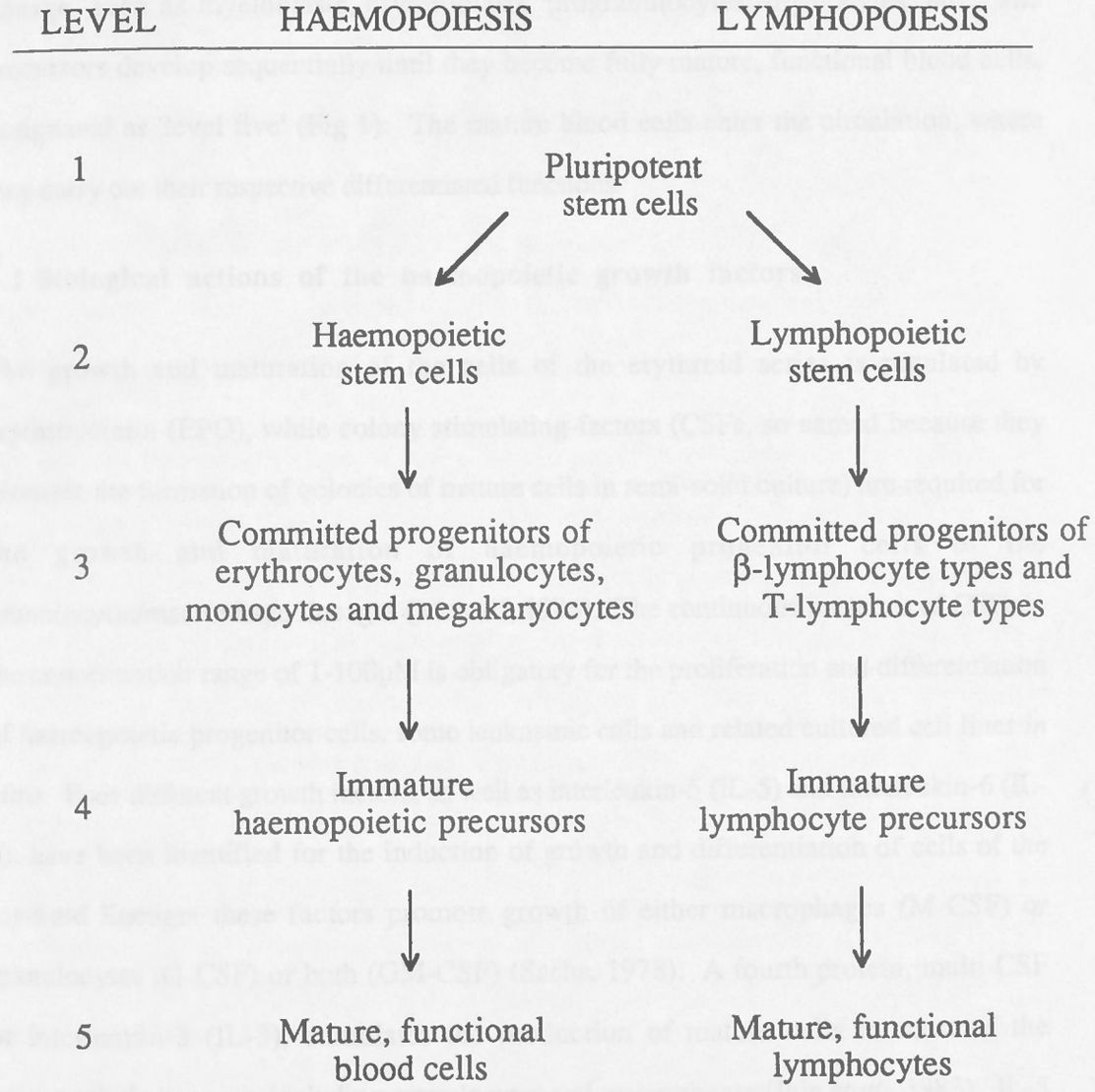
Chapter 1: Introduction

1 Haemopoiesis

Haemopoiesis is the process of progenitor cell proliferation and differentiation that yields terminally-differentiated red cells, neutrophils, eosinophils, basophils, monocytes and platelets in mammals (Watt and Visser, 1992). Although it is a continuous process, one can consider haemopoiesis as having five major maturation levels (Fig 1). The first and most primitive level is that of the pluripotent stem cell. Cells are recruited from this pool to differentiation pathways; others proliferate, leading to the replacement of recruited cells so that the cell pool is perpetuated. This capability of the stem cells is referred to as *self-renewal*. Most cellular systems contain, along with the great majority of differentiated cells with specific functions, a few undifferentiated stem cells that retain their capacity for self-renewal and proliferation. During embryogenesis, most systems depend on the continuous renewal of cells from the primitive stem cell pool whilst, in adult life, only a few tissues retain this dependency. In liver, the stem cells are 'dormant' and only become activated after major cellular depletion, such as that which occurs following partial hepatectomy. On the other hand, stem cells responsible for the renewal of skin, gastrointestinal epithelium, lymphoid tissue and marrow, remain active throughout life. In healthy individuals, lymphopoiesis and haemopoiesis appear to be sustained from two separate cell compartments. This constitutes the second level of haemopoiesis (Fig 1). The common haemopoietic stem cell pool provides progenitors for the six types of blood cells, and the common lymphopoietic stem cell pool gives rise to the various T-lymphocyte and B-lymphocyte subclasses. In the case of haemopoiesis, the progenitor cells which become committed unipotential cells specific for each of the six blood cell types represent the third level of haemopoiesis (Fig 1). The committed progenitor cells which proliferate into visible colonies in a viscous culture medium are referred to as *colony-forming units* (CFU), with a suffix identifying the particular cell line, (e.g. CFU-N for the cell that generates neutrophil colonies). Cells at this level of haemopoiesis are referred to by many authors as *committed stem cells*. Since they show very limited capability for self-renewal, the term *progenitor cells* has been suggested as a better designation. The progenitor cells develop into the precursors which are designated as

Fig 1:- The haemopoietic process occurring in healthy human beings.

Fig 1


LEVEL
HAEMOPOIESIS
LYMPHOPOIESIS
1
Pluripotent stem cells
2
Haemopoietic stem cells
Lymphopoietic stem cells
3
Committed progenitors of erythrocytes, granulocytes, monocytes and megakaryocytes
Committed progenitors of β-lymphocyte types and T-lymphocyte types
4
Immature haemopoietic precursors
Immature lymphocyte precursors
5
Mature, functional blood cells
Mature, functional lymphocytes

'level four' of haemopoiesis (Fig 1). The designation *precursor* has been suggested for those marrow cells that are morphologically identifiable as being part of a specific cell lineage, such as myeloblasts, erythroblasts, progranulocytes, myelocytes, etc. The precursors develop sequentially until they become fully mature, functional blood cells, designated as 'level five' (Fig 1). The mature blood cells enter the circulation, where they carry out their respective differentiated functions.

1.1 Biological actions of the haemopoietic growth factors

The growth and maturation of the cells of the erythroid series is regulated by erythropoietin (EPO), while colony stimulating-factors (CSFs, so named because they promote the formation of colonies of mature cells in semi-solid culture) are required for the growth and maturation of haemopoietic progenitor cells of the granulocytic/macrophage lineages (Metcalf, 1984). The continuous presence of CSFs in the concentration range of 1-100pM is obligatory for the proliferation and differentiation of haemopoietic progenitor cells, some leukaemic cells and related cultured cell lines *in vitro*. Four different growth factors, as well as interleukin-5 (IL-5) and interleukin-6 (IL-6), have been identified for the induction of growth and differentiation of cells of the myeloid lineage: these factors promote growth of either macrophages (M-CSF) or granulocytes (G-CSF) or both (GM-CSF) (Sachs, 1978). A fourth protein, multi-CSF or interleukin-3 (IL-3), stimulates the production of mature cells in most of the haemopoietic lineages, including granulocytes and macrophages (Ihle *et al.*, 1983). IL-5 is a pleitropic cytokine which, in the myeloid series, stimulates the growth of eosinophils selectively. Other factors known to induce eosinophil differentiation are GM-CSF and IL-3, but these activities, which give rise to eosinophils along with other cell types, are not specific to the eosinophil lineage. Little is known about the biology of IL-6 or its action on haemopoietic cells, but it can stimulate the growth of haemopoietic colonies composed of granulocytes and macrophages (Wong *et al.*, 1988). It also stimulates the ability of stem cells to respond to IL-3 and, when combined with other recombinant haemopoietic factors, can augment, or even modify, stem cell responsiveness to them

(Rennick *et al.*, 1989). When added to certain myeloid leukaemia cell lines *in vitro*, IL-6 is also a potent inducer of differentiation (Chieu *et al.*, 1989). Another cytokine [interleukin-1 (IL-1)], known previously as haemopoietin-1, also plays an indirect role in haemopoiesis, mostly by stimulating its target cells to respond to different growth factors (Stanley *et al.*, 1986). Many of the physiological consequences of IL-1 action are mediated, indirectly, through the induction of IL-6 production in cells of the myelopoietic and lymphopoietic series. All of the CSFs, as well as IL-5 and IL-6, are glycoproteins of approximate molecular weights 20-90 KD that stimulate cell proliferation at concentrations of 10^{-11}-10^{-13} M (Table 1; Metcalf, 1989).

Cytokines with growth factor activity are the products of many cell types, including endothelial cells, fibroblasts, stromal cells and some epithelial cells as well as monocytes and T and B lymphocytes (Metcalf, 1988). Some cells have the capacity to synthesize more than one type of CSF simultaneously. Under basal conditions, CSF production is low and, in some cases, the molecules may be retained at or near the cell membrane rather than being released directly into the circulation. CSF production usually rises after inductive signalling such as that which occurs when the levels of more than one type of CSF are elevated during acute infections. The actions of growth factors on the proliferation and differentiation of cells of the myeloid series are presented in Fig 1.1.

CSFs are unrelated at the amino acid sequence level. Despite their structural dissimilarities, they exhibit common functional activities. They stimulate proliferation by acting directly on the progenitor cells, the concentrations of the CSFs determining the length of the cell cycle and the number of progeny cells produced (Metcalf, 1988). In addition they maintain the integrity of the plasma membrane, which ensures cell survival, and they stimulate the functional activities of mature cells. They also commit the entry of bipotential precursor cells into a restricted pathway of exclusive granulocyte or macrophage formation (Metcalf, 1988).

Growth factors act by binding to specific membrane receptors expressed on mature cells as well as those present on progenitor cells at various stages of differentiation (Metcalf,

Table 1: The haemopoietic growth factors.

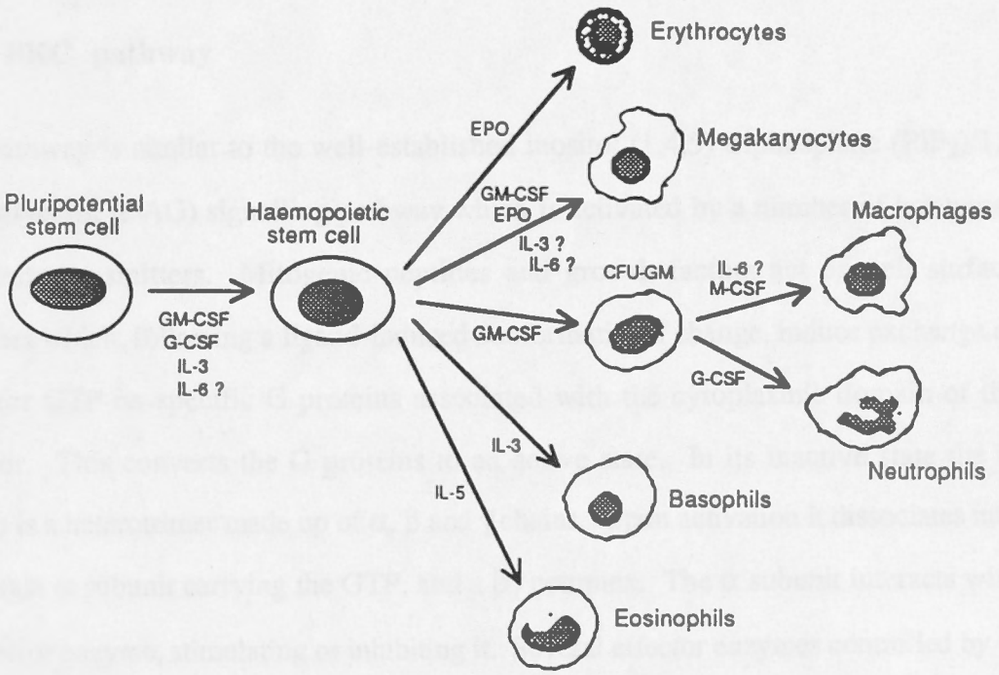
Name	Acronyms	Molecular weight *(M_r)	*Chromosomal location of gene
Granulocyte-macrophage colony-stimulating factor	GM-CSF	14.7	5
Granulocyte colony stimulating factor	G-CSF	18.6	17
Macrophage colony-stimulating factor	M-CSF	21.0	5
Interleukin-3 (Multipotential colony-stimulating factor)	IL-3 (Multi-CSF)	15.4	5
Interleukin-1	IL-1	#10-30	2
Interleukin-5	IL-5	22	5
Interleukin-6	IL-6	29	7

*M_r values and the chromosomal location of the gene are for the human haemopoietic growth factors. #The M_r value depends on the source of the tissue. The molecular weights of the glycoprotein components of the growth factors vary according to tissue source due to variable carbohydrate content.

Fig 1.1:- Role of growth factors in the development of myeloid cells from pluripotential stem cells.

Abbreviations used: GM-CSF, granulocyte macrophage colony stimulating factor; G-CSF, granulocyte colony stimulating factor; EPO, erythropoietin; IL-3, interleukin-3; IL-5, interleukin-5; IL-6, interleukin-6; CFU-GM, colony forming unit, granulocyte and macrophage. (Clark and Kamen, 1987).

Fig 1.1


Erythrocytes
EPO
Megakaryocytes
GM-CSF
EPO
IL-3 ?
IL-6 ?
Pluripotential stem cell
Haemopoietic stem cell
CFU-GM
IL-6 ?
M-CSF
Macrophages
GM-CSF
G-CSF
IL-3
IL-6 ?
GM-CSF
G-CSF
IL-3
IL-5
Basophils
Neutrophils
Eosinophils

1984). The intracellular pathway of signal transduction from the binding of the CSF to the membrane receptor to the integrated cellular response (growth and differentiation) is mediated by two of the most studied signalling pathways: serine/threonine-specific protein kinase C (PKC), and the 'catalytic' receptors containing intrinsic tyrosine-specific protein kinase (RTK) activity.

1.1.1 PKC pathway

This pathway is similar to the well-established inositol (1,4,5) triphosphate (PIP_2)/1,2 diacylglycerol (DAG) signalling pathway which is activated by a number of hormones and neurotransmitters. Mitogenic peptides and growth factors act on cell surface receptors which, following a ligand-induced conformational change, induce exchange of GDP for GTP on specific G proteins associated with the cytoplasmic domain of the receptor. This converts the G proteins to an active state. In its inactive state the G protein is a heterotrimer made up of α, β and γ chains. Upon activation it dissociates into a separate α subunit carrying the GTP, and a $\beta\gamma$ complex. The α subunit interacts with an effector enzyme, stimulating or inhibiting it. Several effector enzymes controlled by G proteins have been described: they include adenyl cyclase (Gilman, 1984), phospholipases (PI-PLC, PLA_2) (Cockcroft, 1987; Burch, 1989; Gupta *et al.*, 1990), and ion channels (Yatani *et al.*, 1988). The mechanism of growth factor signal transduction involves the activation of phosphoinositol-specific phospholipase C (PLC). At least thirteen different forms of PLC have so far been identified in four different PLC gene classes (PLC-α, β, γ and δ). PLC-γ1 is probably the isoenzyme responsible for the hydrolysis of PIP_2 involved in growth factor signalling (Kirz, 1990). The resulting increase in DAG and cytoplasmic Ca^{2+} activates PKC. The substrates for phosphorylation by PKC have not been identified conclusively but they include growth factor receptors, extranuclear and nuclear proto-oncogene products. The signal from the heterotrimeric G protein is turned off by the intrinsic GTPase activity of the α subunit, leading to $\alpha\beta\gamma$ subunit reassociation.

The importance of the PKC pathway in mediating direct effects on cellular proliferation through growth factor receptors is still controversial (Hill *et al.*, 1990; Tucker *et al.*, 1989), but it is possible that modulation of the activities of other growth factor signalling pathways by PKC may be important for cellular proliferation.

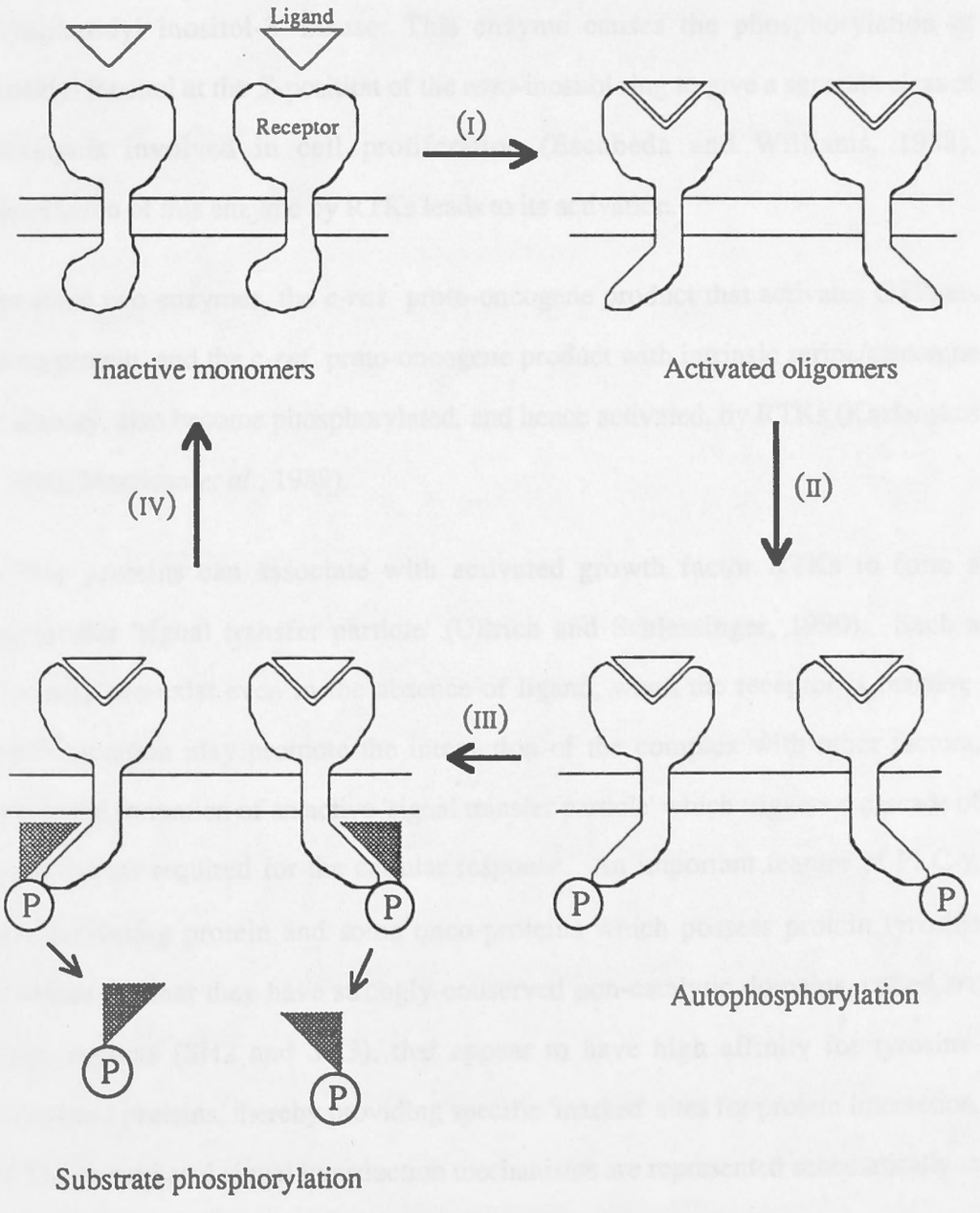
1.1.2 RTK pathway

Growth factor receptors with intrinsic protein tyrosine kinase activity have a similar molecular structure and regions of shared homology. They all possess a large glycosylated extracellular ligand-binding domain, a single hydrophobic transmembrane region, and a cytoplasmic domain that contains tyrosine kinase catalytic activity (Hanks *et al.*, 1988; Yarden and Ullrich, 1988; Schlessinger, 1988). Binding of the growth factor to its receptors causes conformational alteration of the extracellular domain, leading to receptor oligomerization and stabilization of the interactions between adjacent cytoplasmic domains that lead to activation of tyrosine kinase activity (Ullrich and Schlessinger, 1990). In this way, receptor oligomerization permits the transmission of a conformational change from the extracellular domain to the cytoplasmic domain, activating its tyrosine kinase activity to catalyze autophosphorylation and phosphorylation of various cellular substrates. The autophosphorylation of the receptor increases the V_{max} of the kinase activity, maintaining the kinase in an activated state even in the absence of the binding ligand. Receptor autophosphorylation could proceed by an intramolecular or an intermolecular mechanism. In an intramolecular process, the catalytic domain of the receptor molecule phosphorylates specific tyrosine residues located on the same molecule. Alternatively, the catalytic domain of one receptor may phosphorylate specific tyrosine residues on neighbouring receptor molecules. Hence, oligomerization provides a mechanism for receptor activation and also sets the stage for receptor autophosphorylation. Fig 1.2 summarizes the current concepts concerning the molecular steps involved in activation of the receptor tyrosine kinases. Substrates that are phosphorylated by growth factor RTKs include:

Fig 1.2:- Molecular steps involved in the activation of tyrosine-specific protein kinase.

(1) The binding of ligand to the receptor stabilizes an oligomeric state with enhanced protein tyrosine kinase activity; (11) The activated receptor undergoes autophosphorylation; (111) the cytoplasmic domain of the autophosphorylated receptor is more accessible to intracellular substrates, which compete for the substrate-binding region of protein tyrosine kinase; (1V) A tyrosine-specific phosphatase removes the phosphate groups from the receptors, returning the receptor to its original inactive form.

Fig1.2


Ligand
Receptor
(I)
Inactive monomers
Activated oligomers
(II)
(III)
(IV)
P
Autophosphorylation
Substrate phosphorylation

(i) PLC-γ: It is not known whether this is the same PLC-γ as that involved in the PKC signalling pathway. Tyrosine kinase activates PLC-γ by phosphorylating a tyrosine residue on PLC-γ which results in PIP_2 hydrolysis (Wahl *et al.*, 1989).

(ii) Phosphatidyl inositol-3' kinase: This enzyme causes the phosphorylation of phosphatidyl inositol at the 3'-position of the myo-inositol ring to give a separate class of phospholipids involved in cell proliferation (Escobeda and Williams, 1988). Phosphorylation of this enzyme by RTKs leads to its activation.

Besides these two enzymes, the *c-ras* proto-oncogene product that activates GTPase-activating protein, and the c-*raf* proto-oncogene product with intrinsic serine/threonine kinase activity, also become phosphorylated, and hence activated, by RTKs (Kazlauskas *et al.*, 1990; Morrison *et al.*, 1989).

These four proteins can associate with activated growth factor RTKs to form a multimolecular 'signal transfer particle' (Ullrich and Schlessinger, 1990). Such a complex may pre-exist even in the absence of ligand, when the receptor is inactive. Receptor activation may promote the interaction of the complex with other factors, resulting in the formation of an active 'signal transfer particle' which triggers a cascade of molecular events required for the cellular response. An important feature of PLC-γ, GTP-ase activating protein and some onco-proteins which possess protein tyrosine kinase activity is that they have strongly-conserved non-catalytic domains, called *src* homology regions (SH2 and SH3), that appear to have high affinity for tyrosine-phosphorylated proteins, thereby providing specific 'marked' sites for protein interaction. Growth factor-mediated signal transduction mechanisms are represented schematically in Fig 1.3.

The actions of all growth factors on haemopoietic cells lead to the stimulation of cellular proliferation coupled to differentiation. Biochemical evidence indicates that the same molecules (e.g. growth factors) frequently use identical signalling pathways to initiate and carry out completely different responses (e.g. proliferation/differentiation) in

different target cells. Conversely, a single growth factor can stimulate proliferation or differentiation by triggering more than one signalling pathway that may become active subsequently, synergistically or independently, in a particular target cell. The different incoming signals are received by the transcriptional machinery of the target cell which controls the level and diversity of gene expression. Within minutes of receptor stimulation, and without need for protein synthesis, 'immediate early genes' are transcribed (e.g. *c-fos, c-jun*, etc) (Schonthal, 1990). These genes code for nuclear proteins that are involved in transcriptional regulation. The expression of these genes is controlled by transcriptional factors that are present as stable proteins in the cytoplasm (e. g. serum response factors). It is the interaction of the newly synthesized transcriptional regulator with these stable proteins which seems to initiate a 'second wave' of gene expression which, through phosphorylation/dephosphorylation mechanisms, for example, decides the outcome of the 'early signals' received by the cells in response to the binding of the receptor-ligand complex (Nathans *et al.*, 1988; Schonthal, 1990). The result is distinctive: proliferation or differentiation (Fig 1.3).

1.2 Differentiation models

The mechanism of commitment and terminal differentiation of the cells of the haemopoietic system is not well understood. Several models have been proposed for the mechanism of stem cell self-renewal and commitment to differentiation. Each model focuses on different aspects of differentiation and they are not necessarily compatible with each other. The 'Stochastic model' developed by Till *et al* . (1964) proposed that the decision of a stem cell to either renew itself or to yield progeny cells that are committed to differentiation is governed by a stochastic (probabilistic) rule. According to this model, every colony-forming cell may either divide and produce two new cells with the capacity to form colonies or it may differentiate and lose the capacity for colony formation, although it may retain the ability to undergo several divisions, producing a number of fully-differentiated descendants. The processes of proliferation and differentiation occur at random in the population of colony-forming cells, implying that

Fig 1.3:- Regulatory proteins and messenger molecules controlled by tyrosine-specific protein kinases and G protein coupled receptors.

The pathways shown are discussed in Sections 1.1.1 and 1.1.2. Abbreviations: AC, adenylyl cyclase; A kinase, cAMP-dependent protein kinase; C kinase, Ca^{2+}/phospholipid dependent protein kinase C; DAG, diacylglycerol; IP_3, inositol (1,4,5) triphosphate; PI-PLC, phosphoinositide phospholipase C; PI-3K, phosphoinositide-3-kinase; GAP, GTP-ase activating protein; SRF, serum response factor; CREB, cyclic AMP responsive element binding protein; *src* and *raf*, proto-oncogene products. *Jak/Tyk* are the proto-onocogene products of the *c-jun* family. *Grb_2* protein can bind to *msos* protein or it can bind to tyrosine kinase substrate (e.g. *shc* which is also a substrate of *src* kinase). This fusion results in the stimulation of guanine nucleotide coupled receptor signalling pathway. Thus *Grb_2* and *msos* acts as a bridge between receptor tyrosine kinases and G protein-coupled receptors signalling pathways.

d by
: AC,
olipid
e; PI-
GTP-
ement
ogene
ind to
fusion
Thus
upled

Fig 1.3

the individual cells within the population are not closely regulated. In this model the random feature of haemopoietic function displayed by the progeny of a single cell has been compared with the decay of radioactive nuclides. If one studies a large number of radioactive atoms, a very regular pattern of decay following an exponential law is observed, while the decay of any particular individual atom occurs at random in an unpredictable fashion. According to this model, in haemopoietic cells it is the population as a whole that is regulated, rather than individual cells, and control mechanisms act by varying the proliferation and differentiation probabilities. Nakahata *et al* . (1982) investigated the self-renewal of stem cells and production of the colony-forming unit granulocyte-erythrocyte-macrophage-magakaryocyte (CFU-GEMM) by replating individual stem cell colonies. The distribution of stem cells in spleen cell colonies of CFU-GEMM approximated the observations of Till *et al.*, and supported the stochastic model of stem cell renewal.

The haemopoietic inductive micro-environment (HIM) model of Trentin *et al* . (1970), which is based on a sequential cytochemical examination of spleen cell colonies, proposes that the commitment of pluripotent stem cells to monopotent progeny is determined by a specific inductive environment surrounding each individual stem cell. Nakahata *et al.* (1982) observed that upon replating onto a new culture dish a single haemopoietic blast cell colony gives rise to heterogenous progeny capable of multilineage differentiation. This observation suggests that the microenvironment is not obligatory for stem cell commitment.

The third plausible model for the commitment of stem cells is the 'Stem cell competition' model of Van Zant and Goldwasser (1977; 1979). They proposed that humoral factors such as erythropoietin (EPO) and colony-stimulating factors (CSF) play an active role in selecting stem cells for commitment. Metcalf and Johnson (1979) failed, however, to observe competition between EPO and CSF for the selection of individual stem cells in culture. Johnson (1981) observed that all multilineage haemopoietic colonies in culture contain erythroid cells and proposed that the retention of the capacity for erythropoiesis is

an obligatory step in stem cell differentiation. According to this model, except for the erythropoietic lineage, other differentiation potentials are lost successively. Nakahata and Ogawa (1982) identified a haemopoietic colony without erythroid cells that appeared to be terminally-differentiated in only the granulocyte, macrophage and megakaryocyte (GMM) lineages. The identification of such colonies suggests that the processes which govern differentiation of the pluripotent stem cells may be more complex than those proposed by Johnson.

The fifth, and perhaps the most compelling, model has been put forward by Brown *et al.* (1985). This model proposes that lineage potentials are expressed consecutively and individually, in a defined sequence, as progenitor cells mature. It is based on evidence provided by differentiation-resistant HL60 cells. The variant lines can be arranged in a sequence which suggests that HL60 cells first acquire the ability to be induced to differentiated neutrophils, after which they can differentiate into either neutrophils or monocytes or, finally, to monocytes only. Brown *et al.* proposed that, as pluripotent stem cells become committed to differentiation, their features are expressed individually and sequentially in an order determined essentially within the genome. The cells are precommitted with respect to their ability to respond to inducers of differentiation, and, when they encounter the appropriate factors, they undergo proliferation and differentiation along a particular pathway. Progenitor cells which fail to receive a signal for differentiation along a specific pathway automatically progress to the next stage in the sequence of development.

The most recent model of stem cell proliferation and differentiation is that proposed by Moore (1991). According to Moore, the haemopoietic stem cells (HSC) are quiescent under steady-state conditions, which possibly reflects an absence of positive signals or the down-modulation of receptors for these signals. As soon as stem cells enter the cell cycle, they attach themselves [via a specific cytoadhesion molecule (CAM)] to stromal cells. This allows the *c-kit* receptors (which possess intrinsic tyrosine protein kinase activity) to interact with the stromal cell surface proteins commonly known as KL. As a

result, the cells in close proximity to the stem cells (such as endothelial cells, fibroblasts and macrophages) are stimulated to produce positive growth and differentiation factors. Enhanced transcription and/or translation of multiple cytokine genes gives rise to a cytokine cascade involving IL-1 and tumour necrosis factor (TNF). Negative signals for stem cell proliferation which counterbalance the positive stimuli include transforming growth factor β (TGFβ), produced by stromal cells, and interferons, macrophage inflammatory protein-1 (MIP-1) and TNF produced by macrophages. The extracellular matrix, which facilitates the adhesion of stromal cells, can specifically bind, concentrate and present specific cytokines. This model is presented schematically in Fig 1.4.

1.3 Myeloid differentiation and leukaemia

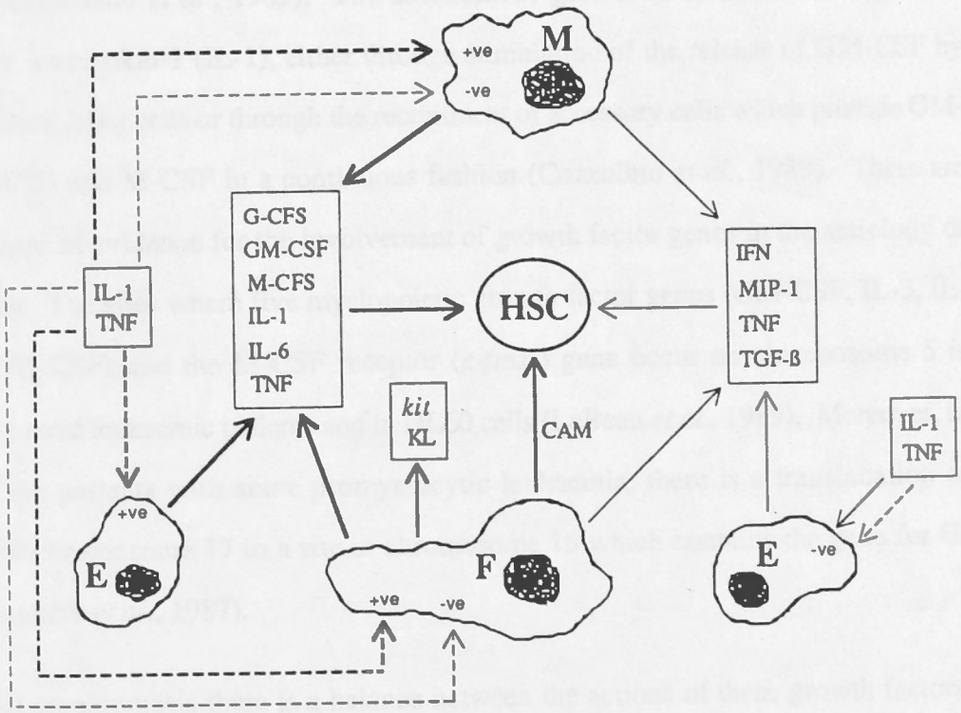
In normal haemopoiesis, the proliferation and differentiation of immature cells is correctly balanced and tightly-regulated. Leukaemia, on the other hand, occurs as a result of changes in the normal development program which can shift the balance towards rapid cell growth. The block in myeloid cell differentiation which characterizes leukaemia occurs because of malignant transformation at the stem cell or progenitor cell level. Leukaemic cells do not differentiate to become functional mature cells, but remain in the proliferative pool and accumulate rapidly (Hassan, 1988). Leukaemic cells fail to differentiate because of an alteration at the level of specific genes, or their products, that are required for differentiation. The defect in differentiation may result from both constitutive expression of proto-oncogenes and the lack of expression of cellular oncogenes (Imazumi *et al.*, 1987). The most striking feature of leukaemic cells is their partial or complete failure to differentiate because of an imbalance in the regulatory events that control the self-renewing and differentiating programs. Leukaemia, therefore, is not so much the result of accelerated cell proliferation but of a failure to limit the size of the pool of proliferative cells via their removal by cell differentiation (Lubbert and Koeffler, 1988).

The development of leukaemia occurs as a result of two sets of genetic changes in the myeloid progenitor cells:

Fig 1.4:- Stem cell proliferation and differentiation model as proposed by Moore (1991).

HSC, haemopoietic stem cell; CAM, cytoadhesion molecule; F, fibroblast cell; E, endothelial cell; M, macrophage; MIP-1, macrophage inflammatory protein-1; TGF β, transforming growth factor β; TNF, tumour necrosis factor; IL-1, interleukin-1; IL-6, interleukin-6; KL, stromal cell surface protein; GM-CSF, granulocyte macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; and IFN, interferon; TNF, tumour necrosis factor. Dark arrows indicate positive signals; light arrows indicate negative signals; dark dotted lines indicate a positive inducing effect and light dotted lines indicate a negative inducing effect.

Fig 1.4


M
+ve
-ve
G-CFS
GM-CSF
M-CFS
IL-1
IL-6
TNF
IL-1
TNF
HSC
IFN
MIP-1
TNF
TGF-ß
kit
KL
CAM
IL-1
TNF
E
+ve
F
+ve
-ve
E
-ve

(1) One set reduces or eliminates the need of the transforming cell for an external supply of growth factors and makes it capable of autonomous growth. Certain strains of leukaemic cells need much lower concentrations of growth factors than normal cells, whilst other strains produce their own growth factors and sustain growth under autocrine stimulation (Metcalf, 1989). Recently, cells from some patients with acute leukaemia have been reported to produce GM-CSF constitutively (Young *et al.*, 1987). In most cases of acute leukaemia, IL-1 has also been shown to be produced in an autocrine fashion (Cozzolino *et al.*, 1989). The autonomous growth in leukaemia is regulated mostly by interleukin-1 (IL-1), either through stimulation of the release of GM-CSF by the leukaemic blast cells or through the recruitment of accessory cells which provide GM-CSF, G-CSF and M-CSF in a continuous fashion (Cozzolino *et al.*, 1989). There are several lines of evidence for the involvement of growth factor genes in the aetiology of leukaemia. The sites where five myelopoietic growth factor genes (GM-CSF, IL-3, IL-4, IL-5, M-CSF) and the M-CSF receptor (*c-fms*) gene occur on chromosome 5 is deleted in most leukaemic patients and in HL60 cells (LeBeau *et al.*, 1989). Moreover, in most of the patients with acute promyelocytic leukaemia, there is a translocation of portion of chromosome 17 to a site in chromosome 15 which contains the gene for G-CSF (Simmers *et al.*, 1987).

In normal myelopoiesis there is a balance between the actions of these growth factors. Deletion of several growth factors and their receptor genes may disturb this balance. A myeloid cell which does not produce differentiation factors in response to growth inducers would show deregulated growth since it could not become committed to maturation. On the other hand, a leukaemic cell which remains capable of producing differentiation factors might not be able to respond to them because it lacks the membrane receptors that transduce the extracellular signals. This view is supported by observations on mouse myeloid leukaemic cell differentiation (Lotem and Sachs, 1986). These cells can be induced to mature when incubated with a protein called macrophage granulocyte inducer (MGI) that induces normal myeloid cell differentiation. Their maturation-associated responses include the up-regulation of a number of membrane receptors for the

growth factor GM-CSF and enhanced production and secretion of this growth factor which then saturates the up-regulated receptors.

(2) The second set of genetic changes uncouple cell growth from differentiation. The cell becomes leukaemic due to uncoupling of the requirement for growth from the requirement for differentiation by changing the expression of the genes which regulate growth from inducible to constitutive. This change asynchronizes the normal differentiation program, resulting in a cell that never differentiates but continues to multiply (Sachs, 1987). Studies with the NFS-60 murine myeloid leukaemic cell line have suggested that the proto-oncogene *c-myb* may be involved in factor-dependent myeloid differentiation and that the shortened *c-myb* gene product may transmit constitutive proliferative signals via IL-3 and G-CSF (Souza *et al.*, 1986). The constitutive expression of the *c-myb* gene also blocks the differentiation of Friend erythroleukaemic cells (Clarke *et al.*, 1988).

The GM-CSF, G-CSF, IL-1 and M-CSF genes are expressed constitutively in some acute leukaemia patients (Oster *et al.*, 1989). A direct link between constitutive GM-CSF and IL-3 expression and leukaemic transformation was provided by transfecting the murine GM-CSF or IL-3 genes into a CSF-dependent nonleukaemic myeloid cell line that subsequently produced leukaemic cells capable of secreting GM-CSF or IL-3 constitutively (Hapel *et al.*, 1986). Hence, uncoupling of the proliferative from the differentiative action of the growth factors may lead to leukaemic transformation. Oncogenes may cause leukaemia by bypassing the requirement that myeloid progenitor cells have for growth factors as proliferative signals by inducing gratuitous tyrosine phosphorylation during signal transduction (Katzav *et al.*, 1989). The uncoupling of the proliferative from the differentiative action of some oncogene products may also lead to leukaemia. The gene product *c-fos* plays a putative role in proliferation as well as in differentiation (Muller and Wagner, 1984), yet in most of the leukaemic cells studied this gene is expressed inappropriately (Miller *et al.*, 1984). Differentiation of HL60 and other leukaemic cells leads to an enhanced expression of *c-fos*, which underlines its association

with functions in the differentiated cells (Collins, 1987; Muller *et al.*, 1982, 1983 and 1984). The imbalance in the expression of certain oncogenes could change gene expression from inducible to constitutive, thereby depriving myeloid cells of their ability to differentiate and condemning them to continuous proliferation. Hence, leukaemic transformation could be the end result of a number of quite different abnormalities. Caution should therefore be exercised in attempting to assimilate all of the existing information into a single model of pathogenesis.

1.4 Classification of leukaemias

The leukaemias have been described according to the predominant cell population, and this criterion for classification has usually been found to correlate with both the clinical course of the disease and its response to treatment. If the major cell population appears to be devoid of differentiated structures, the leukaemia is considered to be *acute*. In the absence of treatment, the survival time of patients with acute leukaemia is measured in weeks. Acute leukaemia is divided into two varieties, *acute lymphoblastic leukaemia* (ALL) and *acute myeloblastic leukaemia* (AML). The presence of granules in the cytoplasm of undifferentiated leukaemic cells and the presence of large metachromatic granules, termed Auer rods, is considered to be characteristic of AML. The diagnosis of ALL is usually made on negative grounds, although histochemical tests [(e.g. Sudan Black and periodic acid Schiff's staining (PAS)] provide supporting evidence for the presence of ALL. The main distinction between these two types of leukaemia is that ALL is common in children, while AML is the predominant form of acute leukaemia in the adult.

When the predominant cellular population is morphologically similar to mature cells, the leukaemia is considered to be chronic and the survival of untreated patients often extends for several years. The classification of this variety of leukaemia presents few problems since the morphological features of the cells are characteristic, and functional abnormalities are observed that represent disturbances of the normal behaviour of the mature cells. Thus, in chronic lymphocytic leukaemia (CLL), large accumulations of

mature lymphocytes are readily identified throughout the body and these are often associated with immunological abnormalities, including immune deficiency and, occasionally, the production of large amounts of gamma globulin. In contrast, in chronic myelogenous leukaemia (CML) mature granulocytes predominate, although their precursors, which are usually restricted to bone marrow, are sometimes found circulating in the peripheral blood. In CML, patients suffer from anaemia, which reflects the disturbed balance between erythropoiesis and granulopoiesis characteristic of this leukaemia. The haemopoietic concept of the two forms of leukaemia is shown in Fig 1.5.

For the last twenty years significant literature has emerged on the differentiation of leukaemic blast/or partially-differentiated leukaemic cells by various agents. Numerous differentiating agents have been discovered, which are used for both *in vitro* and *in vivo* studies. Some of these agents have also been used, and are still being used, in clinical trials for chemotherapy. An anti-leukaemic drug can be used to best effect when its mechanism of action is understood at a fundamental level; the alternative is potentially harmful empirical testing. Many drugs tested during the early days of chemotherapy are now discredited because of their lack of efficacy or their excessive unmanageable toxicity *in vivo*. At a meeting sponsored by the National Cancer Institute (USA) entitled *Conference on a new look at older drugs in cancer treatment: project to review older drugs* (1983), ninety seven of the compounds used in chemotherapy were re-evaluated and only thirteen of these were recommended for further pre-clinical or clinical testing (Hall and Oliverio, 1983). Hence, a detailed knowledge of the mechanism of action of the differentiating agents used in chemotherapeutic practice is essential for their effective usage. The plausible mechanisms of action of some of the differentiating agents used to differentiate leukaemic cells *in vitro* and *in vivo* are discussed below:

1.5 Mechanism of action of differentiating agents on leukaemic cells

The cellular response induced during myeloid differentiation is a continuous process that involves the appearance of two identifiable intermediate states preceding that of terminal

Fig 1.5:- Haemopoiesis in myeloid leukaemia.

The malignant process resides in the stem cell. This pool of cells give rise to erythroid, granulocytic and megakaryocytic precursors. Multiplication of these precursors leads to the accumulation of leukaemic blast cells in marrow. The multiplying blast cells can take one of the two paths: (1) It can become amitotic or sterile and die; or (2) the blast may divide and undergo varying degrees of maturation. A block in maturation at any of these stages leads to leukaemia. 1 shows the path taken by the cells in acute leukamia, while 2 is the path taken by the cells in chronic leukaemia.

ythroid,
ds to the
e one of
vide and
leads to
aken by

Fig 1.5

differentiation (Yen *et al.*, 1984; Yen, 1985). The earliest is the *pre-commitment* state, which can be produced *in vitro* by exposure of cells to an inducer for periods ranging from 20 mins [e.g. phorbol 12-myristate 13-acetate (PMA)-induced differentiation of HL60 cells] to 24 hours [e.g.dimethylsulphoxide (DMSO) and retinoic acid (RA)-induced differentiation of HL60 cells]. In some cases this period corresponds to that of one cell division. Pre-committed cells do not exhibit functional differentiation or G_1/G_0 growth arrest, but they do retain a 'memory' of their initial exposure to the inducer which accelerates their differentiative response to a second exposure. Pre-committed cells can be driven to commitment upon subsequent exposure of the cells to the inducer. Retention of cellular memory has been correlated with structural changes to the nuclear membrane and alterations in the cytosolic calcium- binding anionic proteins (Yen *et al.*, 1984; Yen, 1985). Biochemical changes that occur in the cells during the pre-committed state are reversible, but, once the cell is committed, these changes cannot be reversed (Housman *et al.*, 1978); the cells committed to differentiation proceed to do so despite removal of the inducer. Hence, the behaviour of cells in the pre-committed state is of significance as a 'marker' of the early regulatory state.

There is a variety of agents which induce differentiation of myeloid leukaemic cells by acting at either the pre-committed or committed cellular levels. The action at the pre-committed level includes intervention at both the genomic and signal transduction levels, whilst, once committed, the effect of intervention can be seen at the cell cycle level, the plasma membrane level, the protein synthesis level and the DNA level. Each of these cellular levels is discussed below.

1.5.1 Genomic level

The suppression of *c-myb* (Anfossi *et al.*, 1989) or the *c-myc* gene (Birnie, 1988) may be a crucial factor in the cessation of proliferation. HL60 cells induced to differentiate with a variety of differentiating agents (such as PMA, RA, DMSO, Vitamin D_3, etc.) show suppression of synthesis of *c-myc* mRNA (Collins, 1987). The HL60 TR cells (which are resistant to differentiation by PMA) show suppression of *c-myc* mRNA

levels when treated with PMA but they still fail to express the differentiated phenotype (Collins, 1987). However, when these cells are induced to differentiate into monocytes, by RA + PMA, this is associated with a significant suppression of *c-myb* (Christman *et al.*, 1988). The critical involvement of more than one 'proliferative' oncogene may require more than one blocking agent. The use of multiple differentiating agents which have the capability, collectively, to suppress the expression of *c-myb* and *c-myc* gene products, for example, might lead to a cessation of proliferation associated with persistent suppression of these genes. The available evidence indicates that *c-myc* and *c-myb* code for a group of interacting factors that together fill an essential role in proliferation. Disruption of this interaction by inhibition of the synthesis of one or both of the factors causes the cell to stop proliferation and, simultaneously, to begin differentiation. The expression of the same oncogene is regulated in different ways by different differentiating agents. A significant *decrease* in N-*ras* mRNA synthesis was noted in DMSO-differentiated HL60 cells (Filmus and Buick, 1985), whereas no change in N-*ras* expression was observed in HL60 cells when they were treated with PMA (Sariban *et al.*, 1985). In contrast, the expression of some

cellular oncogenes (e.g. *fos*, *fms*, *src*, and *fgr*) is *enhanced* by differentiating agents. The rapid and transient induction of the *c-fos* gene in PMA and Vitamin D_3-induced differentiation of HL60 cells into macrophages/monocytes is a good example of such positive control (Tsuda *et al.*, 1987). Enhanced *c-fos* expression has also been observed during the differentiation of the mouse cell line WEHI-3B into monocytes as well as in the normal pathway of macrophage/monocyte differentiation (Muller *et al.*, 1984). Although it has been proposed (Birnie, 1988) that the expression of the *c-fos* gene may be involved in the differentiation of HL60 cells, recent evidence suggests that the expression of this gene is neither a sufficient nor necessary aspect of the differentiation process (Mitchell *et al.*, 1986); any role that it may play is presently unclear. Similarly, enhanced *c-fms* mRNA levels (coding for the M-CSF receptor) have also been observed in HL60 cells terminally-differentiated to macrophages/monocytes by PMA and Vitamin D_3, but no such enhancement was observed when cells of the same lineage were

differentiated with granulocytic inducers like DMSO and RA (Sariban *et al.*, 1985). In contrast, the *c-src* gene product, which has intrinsic tyrosine kinase activity, increased when HL60 cells were treated with both granulocytic and macrophage/monocytic inducers (Gee *et al.*, 1986), while the expression of *c-fgr* , which is also associated with enhanced tyrosine kinase activity, was increased markedly only when these cells were triggered with granulocytic inducers (Collins, 1987).

1.5.2 Signal transduction level

The transduction of signals from extracellular ligands which have the capacity to induce differentiation depends, possibly, on the activities of the protein kinases which make up the signal transduction network (Berridge, 1987). The two most studied signalling pathways activated by growth factors and differentiating agents are the serine/threonine-specific protein kinase C (PKC) and the tyrosine-specific protein kinase activities associated with the cytoplasmic domains of 'catalytic' receptors. The mechanisms by which the binding of growth factors and differentiating agents to receptors at the cell surface result in the activation of signal transduction pathways are discussed in Section 1.1.1. Signalling that occurs through G-protein-coupled receptors and through receptor-associated tyrosine kinases are tightly interconnected processes (Ullrich and Schlessinger, 1990). In fact, growth factors and differentiating agents which act through the two pathways usually interact synergistically (Rozengurt, 1986) and the respective enzyme activities are controlled by both types of receptor.

Various agents induce differentiation by signalling through a number of pathways that function sequentially or in parallel via altered activities of protein kinases and phosphatases. PMA-induced differentiation of HL60 cells occurs through activation of the Ca^{2+}/ phospholipid dependent PKC, where PMA itself acts as an analogue of DAG and binds directly to PKC (Collins, 1987). The differentiation of HL60 cells induced by RA is associated with increased activity of both cAMP-dependent protein kinase A and Ca^{2+}/phospholipid-dependent protein kinase C but does not appear to involve the Ca^{2+}/calmodulin-dependent protein kinase (Yen *et al.*, 1986 and Wu, 1989). DMSO and

dimethyl formamide (DMF) both increase cAMP-dependent protein kinase A activity (Fontana *et al.*, 1986) while decreasing that of Ca^{2+}/phospholipid-dependent protein kinase C (Faleto *et al.*, 1985). Whereas DMSO alone increases the activity of a tyrosine-specific protein kinase in a cell free system (Srivasta, 1985), it also increases protein kinase A activity in HL60 cells (Yen *et al.*, 1986). In Friend erythroleukaemic cells, DMSO decreases phosphatidylinositol turnover, possibly by modulating the activity of PLC, under conditions where it also induces differentiation (Faleto *et al.*, 1985). Vitamin D_3-induced differentiation of HL60 cells is accompanied by the activation of a neutral sphingomyelinase that causes the hydrolytic cleavage of sphingomyelin into ceramide and cholinephosphate. In this case, ceramide may be a component of the differentiation process that acts as a second messenger (Okazaki *et al.*, 1989 and 1990).

The same differentiating agent may trigger distinctly different processes in different leukaemic cell types that converge eventually on the activation of a small number of common pathways. The two major signal transduction systems (cAMP-dependent protein kinase A and Ca^{2+}/phospholipid-dependent protein kinase C) evidently cooperate to inhibit growth and induce differentiation of myeloid leukaemic cells. The phosphorylation of target proteins as a consequence of cAMP action is able to antagonize, for example, the effects of the expression of both *c-ras* and *c-src* oncogenes and, thereby, to reverse the malignant transformation produced by them (Puck, 1987).

1.5.3 Cell cycle level

Normal cells can enter a quiescent phase of the cell cycle (G_0) when deprived of essential metabolites. They are able to re-establish cycling when conditions become more favourable. Leukaemic cells have lost this ability due to mutation in at least two genes, one of which leads to unregulated growth (*c-myc* and *c-myb)* whilst the other is associated with loss of a constitutive mitogenic signal (e.g. *c-ras*). Both mutations probably occur independently, causing leukaemic cells to either multiply rapidly or cycle continuously. Most of the differentiating agents operate by arresting growth of these rapidly-proliferating cells at the G_1/G_0 phase or the S phase of the cell cycle. The cellular

program of differentiation and growth arrest at G_1/G_0 is not well understood. DMSO initiates differentiation in leukaemic cells by causing growth arrest at the G_1 phase [i.e. after one cell division cycle has been completed (24 hours)]. RA also induces growth arrest (and, hence, differentiation) at the same phase of the cycle but only after a two-cell division cycle (48 hours) has occurred (Yen *et al.*, 1986). The differentiation induced by PMA, on the other hand, can occur in the absence of any cell division or new DNA synthesis (Collins, 1987). Inhibitors like cytosine-arabinoside and 6-thioguanine induce differentiation by acting at the S-phase of the cell cycle, thereby inhibiting DNA synthesis (Hassan and Rees, 1989).

1.5.4 Plasma membrane and protein synthesis level

Protein synthesis inhibitors like actinomycin-D have been shown to synergize with RA in inducing differentiation of myeloid leukaemic cells (Hassan and Rees, 1989). Differentiation was inhibited by the presence of certain cell surface glycoproteins, leaving open the possibility that the inhibition of glycoprotein synthesis by actinomycin-D might have facilitated differentiation by RA. In the same way, anthracyclines like aclacinomycin A and marcellomycin and antibiotics-like tunicamycin, all of which block the synthesis of membrane glycoproteins, can induce differentiation of HL60 cells (Morin and Sartorelli, 1984; Nakayasu *et al.*, 1980). Some antimetabolites of *de novo* purine and pyrimidine biosynthetic pathways (like mycophenolic acid, 6-mercaptopurine ribonucleotide and acivicin) can also induce differentiation of HL60 cells by indirectly inhibiting the synthesis of membrane glycoproteins (Lucas *et al.*, 1983; Sokoloski and Sartorelli, 1987; Lyons, 1989). These antimetabolites inhibit inosine monophosphate dehydrogenase (IMP dh), the key regulatory enzyme of the *de novo* purine pathway, and thus cause depletion of intracellular GTP pools. Cellular GTP acts as a mediator or a modulator of a wide variety of cellular processes implicated in the regulation of cellular growth and differentiation (Hughes, 1983). One such role involves the activation of both fucosyl and mannosyl units to their corresponding GDP-nucleotide sugars as an obligatory step in their utilization for glycoprotein synthesis (Sokoloski and Sartorelli,

1986). The synthesis of glycoprotein involves the formation of oligosaccharide groups attached N-glycosidically to asparagine residues by a process involving the transfer of a preformed dolichol-linked precursor to a nascent peptide acceptor (Kornfeld and Kornfeld, 1980). The formation of the dolichol-linked oligosaccharide, on the other hand, occurs by the sequential addition of mannose residues to growing chains that utilize GDP-mannose and dolichol-mannose (which in turn is formed from GDP-mannose) as the donor molecules. The synthesis of the oligosaccharide structure of the glycoprotein present on the cell surface depends on the metabolic environment within the cell as well as the availability of nucleotide sugar pools, the activation of the necessary enzymes and the availability of the appropriate protein acceptor molecule. Hence, inhibition of IMP dehydrogenase, which regulates the synthesis of GDP, might have the capacity to alter the biosynthesis of glycoproteins indirectly and thereby to alter the behaviour of malignant cells in a manner which initiates differentiation (Sokoloski *et al.*, 1986).

On the other hand, some differentiating agents act by modulating the glycosylation of membrane glycoproteins. DMSO, RA and PMA, for example, all decrease the sulphation of the glycosaminoglycan (GAG) of membrane glycoproteins. Production of this undersulphated GAG could result in the release, from leukaemic cells, of a glycoprotein thought to be responsible for inhibiting differentiation, thereby allowing the cells to differentiate (Lee, 1987). Alternatively, the undersulphated GAG itself (resulting from DMSO, RA and PMA treatment) could, due to its lower polyanionic charge and, hence, weaker binding capability, cause the release of leukaemia-associated inhibitor (LAI), which may in some way initiate differentiation of HL60 cells (Olofosson *et al.*, 1980). In some cases the induction of differentiation is followed by the enhanced synthesis of particular proteins: the enhanced synthesis of *differentiation associated factor* (DAF) by murine erythroleukaemic cells occurs, for example, following their induction with hexamethylenebisacetamide (Sparatore *et al.*, 1991). Differentiating agents also act at the plasma membrane by inhibiting or triggering the synthesis of different cell surface proteins. Differentiation-dependent proteins have been observed on the external surface of the plasma membrane of HL60 cells in response to DMSO and RA (Felsted *et al.*,

1983). At the same time, the synthesis of receptors for transferrin, a protein responsible for the transport of iron, is suppressed by most of the differentiating agents that act on HL60 cells (Collins, 1987). The synthesis of new differentiation proteins and inhibition of the synthesis of transferrin receptors on the surface membranes of HL60 cells both appear to play a critical role in the differentiation mechanism. Moreover, as discussed above, differentiating agents also act on the plasma membrane by altering the synthesis of glycoproteins. In the case of Friend erythroleukaemic cells (Sato *et al.*, 1980), differentiation is induced after treating these cells with DMSO for periods longer than 20 hours, which results in the loss of heparan sulphate from the glycoaminoglycan in the surface membrane. However, when these cells are exposed to DMSO for only 30 mins, and the DMSO removed for 3-4 hours, the glycoaminoglycan lost is completely recovered by biosynthesis owing to the rapid turnover of heparan sulphate in Friend's cells (Sato *et al.*, 1980).

1.5.5 DNA level

Cytosine-arabinoside increases the activity of DNA topoisomerase 11 (Cunningham *et al.*, 1988). RA induced-differentiation, which is dependent on the continuing activity in the cell nucleus of topoisomerase 11-mediated cleavage, is enhanced in the presence of cytosine-arabinoside (Francis *et al.*, 1988). On the other hand, etoposide, which inhibits topoisomerase activity, also inhibits the RA-induced differentiation of HL60 cells to granulocytes (Francis *et al.*, 1988). PMA-induced differentiation of HL60 cells is also partially blocked by inhibitors of topoisomerase 11, suggesting that this DNA-modifying enzyme could be an important target for activated PKC in differentiated cells (Sahyoun *et al.*, 1986). Changes in DNA are also associated with the induction of erythroid differentiation of murine erythroleukaemic cells by DMSO and butyrate (Terada *et al.*, 1978). Such changes may involve DNA cleavage mediated, possibly, through the direct activation of DNAase or by the release of the enzyme from its bound location. Since DMSO is also known to affect membrane fluidity, changes in lysosomal membranes may also be involved in the accessibility of DNAase to its substrate (Bernstein *et al.*, 1976;

Kimhi *et al.*, 1976). DNAase action could produce single-strand nicks in those regions of the chromosome that are required for transcription of specific differentiation products (Scher and Friend 1978). It is also possible that the nicks themselves act as initiation sites for RNA-polymerizing activity, or that the cleavage of DNA might lead to the release of small DNA degradation products that could themselves act as inducers (either directly, or after subsequent metabolism). The level of a known inducer like hypoxanthine, for example, (Guesella and Housman, 1976) may be increased to a concentration at which differentiation would occur. The differentiation of HL60 cells induced by butyrate probably occurs through the inhibition of histone deacetylase, resulting in the hyperacetylation of histone proteins (Kruh, 1982) and associated changes in the chromatin structure which make the DNA more accessible to the action of DNAase (Kruh, 1982). Neplanocin (a cyclopentenyl adenosine) induces differentiation in HL60 cells by inhibiting the methylation of both DNA and RNA (Linevsky *et al.*, 1985). Possible modes of action of the different differentiating agents are presented in Fig 1.6.

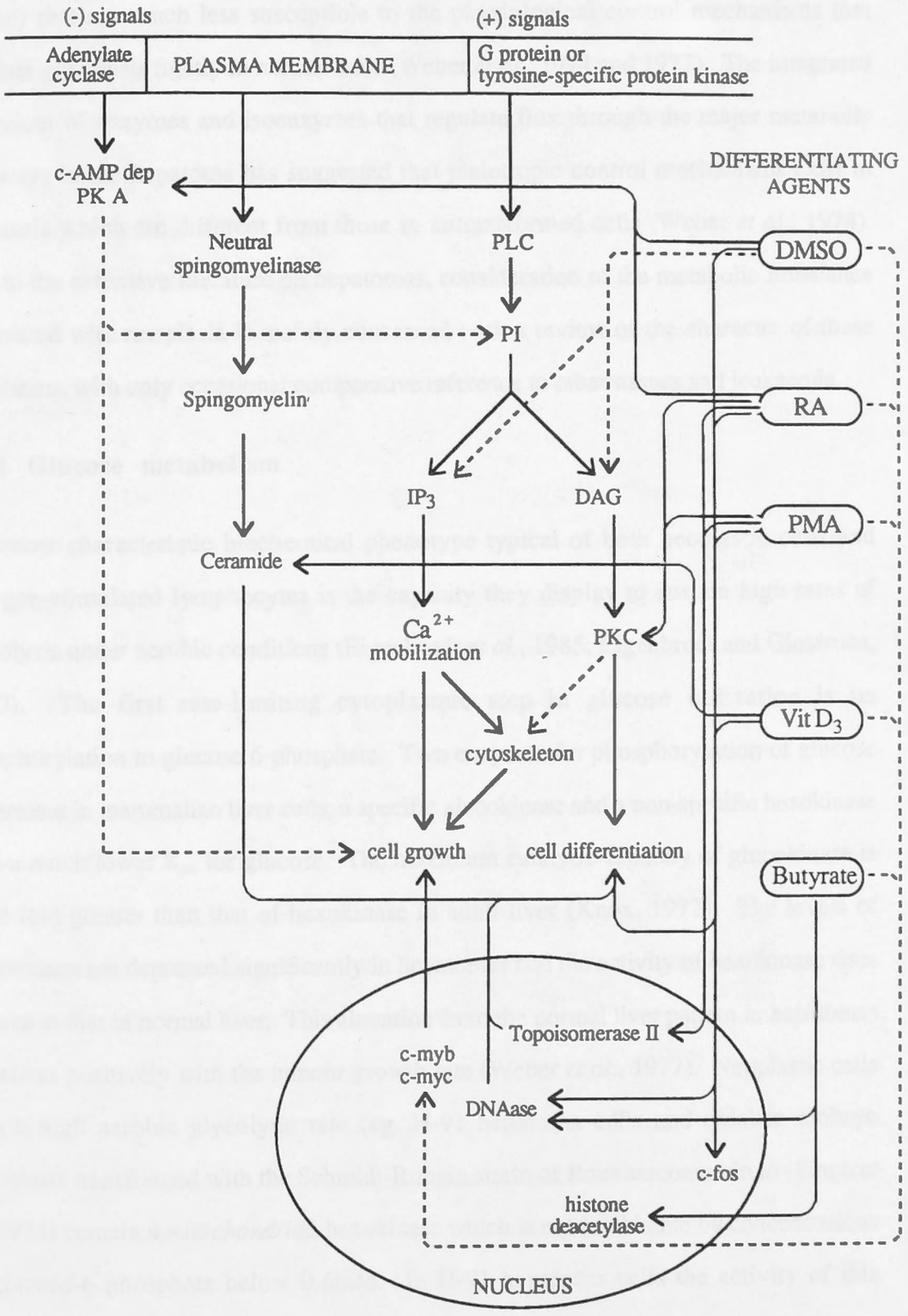
1.6 Metabolic imbalance associated with neoplasia, with special reference to leukaemia

Normal and neoplastic cells have to fulfill certain metabolic prerequisites in order to proliferate. In addition to the supply of energy, an adequate supply of precursors is needed for nucleic acid biosynthesis. In rapidly-proliferating cells (eg. neoplastic cells) the levels and/or the availability of the glycolytic and glutaminolytic intermediates are enhanced to meet the increased demand for low molecular weight substrates for the synthesis of DNA, proteins and other essential macromolecules. The alterations in the metabolic balance that are characteristic of neoplastic transformation suggest that a reprogramming of gene expression has occurred, resulting in the altered behaviour of key enzymes of the major metabolic pathways (especially carbohydrate metabolism, glutamine metabolism and nucleic acid metabolism). This may include a shift in the isoenzyme pattern, like that observed in glycolysis where the disappearance of high K_m regulatory isoenzymes (e.g. glucokinase and L-type pyruvate kinase) is accompanied by

Fig 1.6:- Interaction between differentiating agents in inducing differentiation and inhibition of growth of myeloid leukaemic cells.

PLC, phospholipase C; IP_3, inositol 1,4,5, triphosphate; DAG, diacylglycerol; PI, phosphatidylinositol; RA, retinoic acid; PMA, phorbol myristate acetate; PKC, protein kinase C; PK A, protein kinase A; Vit D_3; Vitamin D_3.

Fig 1.6


(-) signals
(+) signals
Adenylate cyclase
PLASMA MEMBRANE
G protein or tyrosine-specific protein kinase
c-AMP dep PK A
DIFFERENTIATING AGENTS
Neutral spingomyelinase
PLC
DMSO
PI
Spingomyelin
RA
IP3
DAG
PMA
Ceramide
Ca2+ mobilization
PKC
Vit D3
cytoskeleton
cell growth
cell differentiation
Butyrate
Topoisomerase II
c-myb
c-myc
DNAase
c-fos
histone deacetylase
NUCLEUS

an increase in the production of low K_m isoenzymes (e.g. hexokinase, M-type pyruvate kinase) that are much less susceptible to the physiological control mechanisms that regulate glycolysis tightly in normal cells (Weber *et al.*, 1974 and 1977). The integrated behaviour of enzymes and isoenzymes that regulate flux through the major metabolic pathways in the hepatoma has suggested that pleiotropic control mechanisms exist in neoplasia which are different from those in untransformed cells (Weber *et al.*, 1974). Due to the extensive literature on hepatomas, consideration of the metabolic imbalance associated with neoplasia is mainly concerned with a review of the character of these neoplasms, with only occasional comparative reference to other tissues and leukaemia.

1.6.1 Glucose metabolism

The most characteristic biochemical phenotype typical of both neoplastic cells and mitogen-stimulated lymphocytes is the capacity they display to sustain high rates of glycolysis under aerobic conditions (Eigenbrodt *et al.*, 1985; Eigenbrodt and Glossman, 1980). The first rate-limiting cytoplasmic step in glucose utilization is its phosphorylation to glucose 6-phosphate. Two enzymes for phosphorylation of glucose are present in mammalian liver cells, a specific glucokinase and a non-specific hexokinase with a much lower K_m for glucose. The maximum catalytic capacity of glucokinase is three-fold greater than that of hexokinase in adult liver (Knox, 1972). The levels of glucokinase are depressed significantly in hepatomas and the activity of hexokinase rises relative to that of normal liver. This alteration from the normal liver pattern in hepatomas correlates positively with the tumour growth rate (Weber *et al.*, 1977). Neoplastic cells with a high aerobic glycolytic rate (eg. H-91 hepatoma cells and chicken embryo fibroblasts transformed with the Schmidt-Ruppin strain of Rous sarcoma virus) (Singh *et al.*, 1974) contain a *mitochondrial* hexokinase which is not inhibitable by concentrations of glucose-6 phosphate below 0.6mM. In H-91 hepatoma cells the activity of this enzyme is approximately twenty-fold greater than that found in normal liver (Pedersen, 1978). In most cell types hexokinase acts at low glucose concentration to provide immediate energy needs for cell maintenance, while glucokinase appears only in mature

liver cells to capture glucose at higher than normal concentration for storage as glycogen and fatty acids (Longenecker, 1980). Thus, in neoplastic hepatic tissue, the change in enzyme profile suggests that the tissue has an increased capacity to utilize glucose because of its greater potential to phosphorylate it via hexokinase. This is consistent with the decrease in glycogen deposition and the depressed activity of phosphoglucomutase and glycogen synthase that are also observed in neoplastic liver cells (Weber *et al.*, 1977; Hammond and Balinsky, 1978). The high glycolytic activity of neoplastic cells may be due to the combination of an enhanced rate of glucose transport and the presence of elevated activities of unique forms of hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK) (Weber *et al.*, 1974; Eigenbrodt *et al.*, 1985). It has been observed, in parallel, that the increase in hepatoma growth is accompanied by increases in the activities of the key glycolytic enzymes (HK, PFK and PK), while the activities of key gluconeogenic enzymes (glucose 6-phosphatase, fructose 1,6-diphosphatase, phosphoenol pyruvate carboxykinase and pyruvate carboxylase) decrease (Weber *et al.*, 1974). The absence of fructose 1,6-diphosphatase is a characteristic shared by hepatoma cells (Eigenbrodt *et al.*, 1985) and some leukaemic cells. Increased expression of the gene for this enzyme is activated upon differentiation of human leukaemic HL60 cells with Vitamin D_3 (Solomon *et al.*,1988). Normal bone marrow cells also contain low levels of gluconeogenic enzymes, consistent with the low activity of this pathway in cells other than liver and kidney cortex. The data indicates that the expression of genes for these key enzymes of carbohydrate metabolism is linked to the rate of cell proliferation in different neoplastic cell lines. Rapidly-proliferating cells and neoplastic cells contain the pyruvate kinase isoenzyme M_2 (PK-M_2). PK-M_2 is phosphorylated and inactivated by a cAMP-independent protein kinase. This enzyme is allosterically activated by millimolar concentrations of fructose 1,6-diphosphate and 1-pyrophosphorylribosyl 5-phosphate (PRPP). Rapidly proliferating cells, neoplastic cells and virally-transformed cells contain elevated concentrations of fructose 1,6-diphosphate and PRPP (Eigenbrodt *et al.*, 1985). High levels of fructose 1,6-diphosphate enhance the activity of PFK, which leads to more rapid utilization of fructose-6 phosphate and a decrease in the steady-state

concentration of glucose-6 phosphate, resulting in the activation of HK. Elevated fructose 1,6- diphosphate also activates PK, so the coordinated activation of these enzymes leads to high glycolytic rates in neoplasia (Eigenbrodt *et al.*, 1985). Since the activity of fructose 1,6- diphosphatase is strongly reduced or absent in neoplastic cells, fructose 1,6-diphosphate and aerobic glycolysis remain elevated.

Ribose 5-phosphate (Rib 5-P), an important precursor for the biosynthesis of nucleic acids, may be generated either by the direct oxidative pentose phosphate pathway (OPP), involving the action of a rate-limiting, irreversible enzyme [glucose 6-phosphate dehydrogenase (G6PDH)] or by the non-oxidative pentose phosphate pathway, from hexose-6 phosphate and triose phosphate (Williams and Blackmore, 1983). In normal cells, both pathways of pentose phosphate production operate in the direction of Rib 5-P production when the utilization of this intermediate is accelerated by the demands of DNA and RNA synthesis. In all hepatomas, irrespective of their growth rates, the activities of G6PDH and transaldolase (a regulatory enzyme of the non-oxidative pathway) are increased (Weber *et al.*, 1974; Heinrich *et al.*, 1976). This emphasizes the important role of the oxidative and non-oxidative pathways in the synthesis of Rib 5-P. This intermediate is the precursor of PRPP, an indispensable substrate for both the *de novo* and salvage pathways of purine and pyrimidine synthesis (Henderson, 1972; Henderson and Paterson, 1973).

The metabolism of glucose by the non-oxidative pentose pathway reactions occurs in cells by two types of reaction sequence. The classical pathway has been referred to as the 'fat cell' or 'F-type pathway' [Fig 1.7 (a)], since it has been established that this mechanism operates exclusively in rat epididymal fat pad tissues (Williams *et al.*, 1974; Blackmore *et al.*, 1982). The second reaction sequence was found in liver (Williams *et al.*, 1978 a,b) and its existence in photosynthetic tissue was proposed by Clark *et al.* (1974) and a number of its reactions confirmed in the path of CO_2 fixation by spinach chloroplasts *in vivo* and *in vitro* (Kapuscinski and Williams, 1984). This reaction sequence, [Fig 1.7 (b)] is referred to as the L-type (liver type) pentose cycle and it has

Fig 1.7 (a): Reaction sequence of the F-type pentose pathway (now known to occur in adipose tissue).

The enzymes involved in this sequence of reactions are: (e) ribulose 5-phosphate 3-epimerase, (i) ribose 5-phosphate isomerase, (TK) transketolase, (TA) transaldolase, (PHI) phosphohexose isomerase, aldolase. Abbreviations used: Xlu 5-P, Xylulose 5-phosphate; Ru 5-P, Ribulose 5-phosphate; Rib 5-P, Ribose 5-phosphate; Fru 6-P, Fructose 6-phosphate; Gra 3-P, Glyceraldehyde 3-phosphate; Seh 7-P, Sedoheptulose 7-phosphate; Ery 4-P, Erythrose 4-phosphate; Glu 6-P, Glucose 6-phosphate; Fru 1,6 P_2, Fructose 1,6 diphosphate.

Fig 1.7 (a)

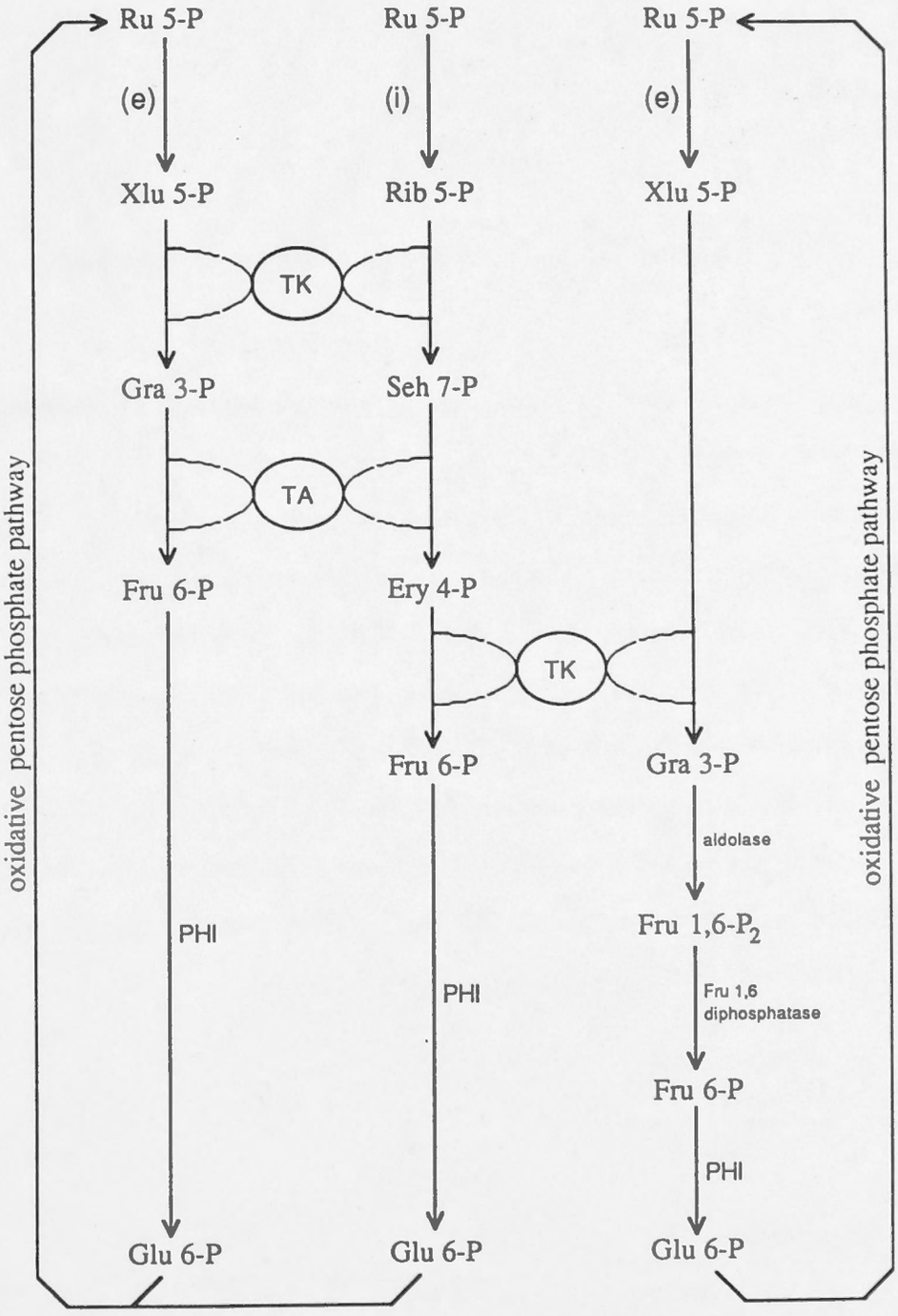
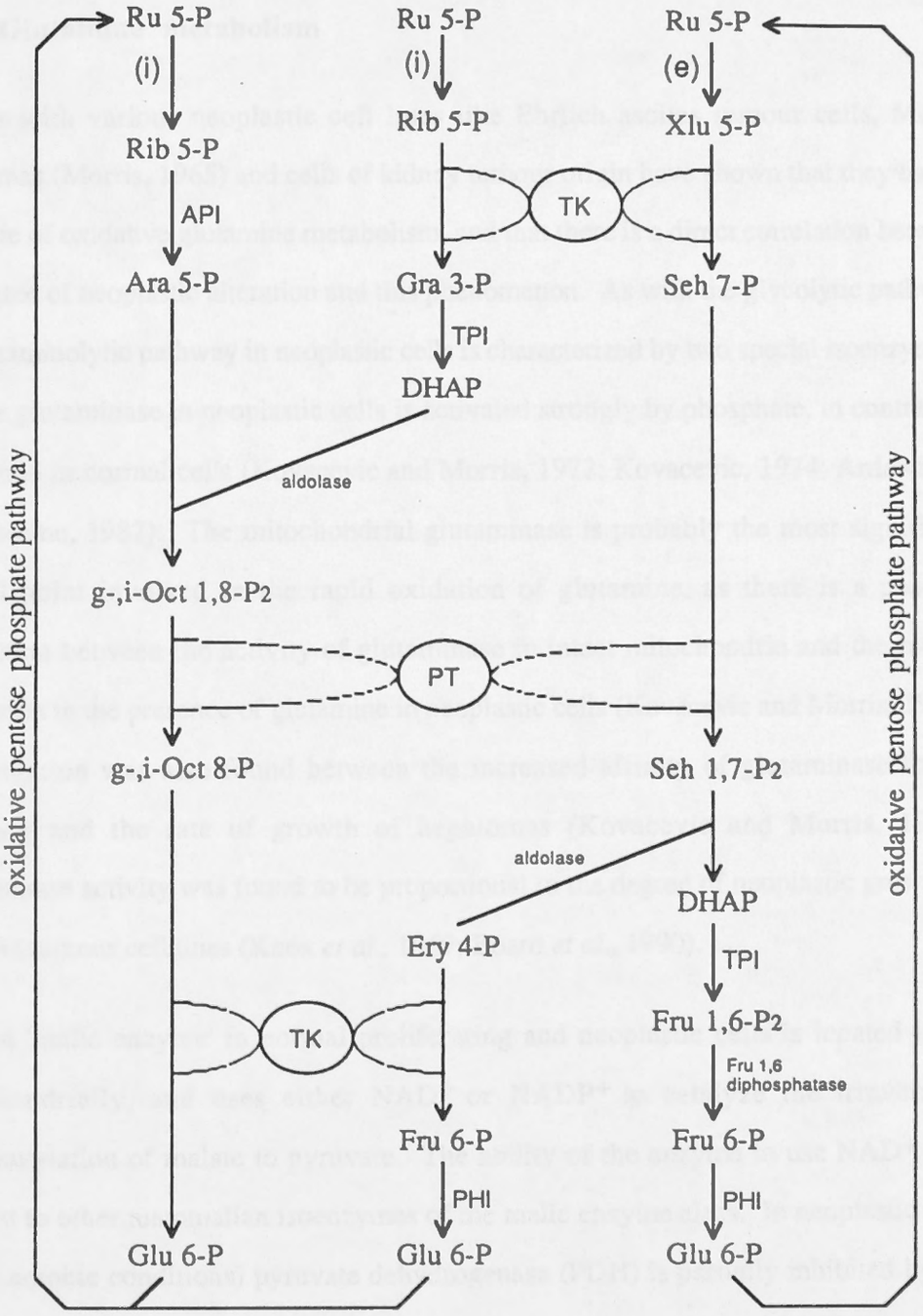

Ru 5-P
Ru 5-P
Ru 5-P
(e)
(i)
(e)
Xlu 5-P
Rib 5-P
Xlu 5-P
TK
Gra 3-P
Seh 7-P
TA
Fru 6-P
Ery 4-P
TK
Fru 6-P
Gra 3-P
aldolase
Fru 1,6-P2
PHI
PHI
Fru 1,6 diphosphatase
Fru 6-P
PHI
Glu 6-P
Glu 6-P
Glu 6-P
oxidative pentose phosphate pathway
oxidative pentose phosphate pathway

Fig 1.7 (b): Reaction sequence of the L-type pentose pathway (now known to occur in liver).

The enzymes involved in the pathway are: (e) ribulose 5-phosphate 3-epimerase, (i) ribose 5-phosphate isomerase, (API) arabinose 5-phosphate ketolisomerase, (TK) transketolase, (TA) transaldolase, aldolase, (TPI) triose phosphate isomerase, (PT) D-glycero D-ido Octulose 1,8 diphosphate: D-altro-heptulose 7-phosphotransferase, (PGI) phosphoglucose isomerase. Abbreviations used: Xlu 5-P, Xylulose 5-phosphate; Ru 5-P, Ribulose 5-phosphate; Rib 5-P, Ribose 5-phosphate; Fru 6-P, Fructose 6-phosphate; Gra 3-P, Glyceraldehyde 3-phosphate; Seh 7-P, Sedoheptulose 7-phosphate; Ery 4-P, Erythrose 4-phosphate; DHAP, Dihydroxyacetone phosphate; Glu 6-P, Glucose 6-phosphate; Ara 5-P, Arabinose 5-phosphate; Seh 1,7-P_2, Sedoheptulose 1,7 diphosphate; g-,i-Oct 8-P, D-glycero D-ido Octulose 8-phosphate; g-,i-Oct 1,8-P_2, D-glycero D-ido Octulose 1,8 diphosphate; Fru 1,6-P_2, Fructose 1,6 diphosphate.

Fig 1.7 (b)


Ru 5-P
(i)
Rib 5-P
API
Ara 5-P
Ru 5-P
(i)
Rib 5-P
Gra 3-P
TPI
DHAP
Ru 5-P
(e)
Xlu 5-P
Seh 7-P
TK
aldolase
g-,i-Oct 1,8-P2
PT
g-,i-Oct 8-P
Seh 1,7-P2
aldolase
DHAP
Ery 4-P
TPI
Fru 1,6-P2
TK
Fru 1,6
diphosphatase
Fru 6-P
Fru 6-P
PHI
PHI
Glu 6-P
Glu 6-P
Glu 6-P
oxidative pentose phosphate pathway
oxidative pentose phosphate pathway

been shown to be active in liver and many neoplasms of varying growth rates (Arora, 1984).

1.6.2 Glutamine metabolism

Studies with various neoplastic cell lines like Ehrlich ascites tumour cells, Morris hepatomas (Morris, 1965) and cells of kidney tumour origin have shown that they have a high rate of oxidative glutamine metabolism, and that there is a direct correlation between the degree of neoplastic alteration and this phenomenon. As with the glycolytic pathway, the glutaminolytic pathway in neoplastic cells is characterized by two special isoenzymes : (1) The glutaminase in neoplastic cells is activated strongly by phosphate, in contrast to that found in normal cells (Kovacevic and Morris, 1972; Kovacevic, 1974; Ardawi and Newsholme, 1982). The mitochondrial glutaminase is probably the most significant control point involved in the rapid oxidation of glutamine, as there is a positive correlation between the activity of glutaminase in intact mitochondria and the rate of respiration in the presence of glutamine in neoplastic cells (Kovacevic and Morris, 1972). A correlation was also found between the increased affinity of glutaminase for its substrate and the rate of growth of hepatomas (Kovacevic and Morris, 1972). Glutaminase activity was found to be proportional to the degree of neoplastic growth of different tumour cell lines (Knox *et al.*, 1969; Board *et al.*, 1990).

(2) The 'malic enzyme' in normal proliferating and neoplastic cells is located intra-mitochondrially, and uses either NAD^+ or $NADP^+$ to catalyze the irreversible decarboxylation of malate to pyruvate. The ability of the enzyme to use NAD^+ is in contrast to other mammalian isoenzymes of the malic enzyme class. In neoplastic cells (under aerobic conditions) pyruvate dehydrogenase (PDH) is partially inhibited by the production of superoxide formed from oxygen (Eigenbrodt *et al.*, 1985). Furthermore, the extramitochondrial pyruvate generated via the glycolytic pathway is not transported efficiently to mitochondrial PDH and hence is reduced to cytoplasmic lactate. PDH inside the mitochondrion utilizes preferentially the pyruvate produced in the mitochondrial matrix from glutamine via the $NADP^+$-linked malic enzyme. Hence, in neoplastic cells,

the NAD(P)-linked malic enzyme is an essential component of the pathway responsible for the complete oxidation of glutamine. Regulation of its activity may be necessary to allow the generation of precursors at a sufficient rate to meet the anabolic demands of neoplastic cells. The pathways of glucose and glutamine metabolism in neoplastic cells are presented in Fig 1.8.

1.6.3 Lipid metabolism

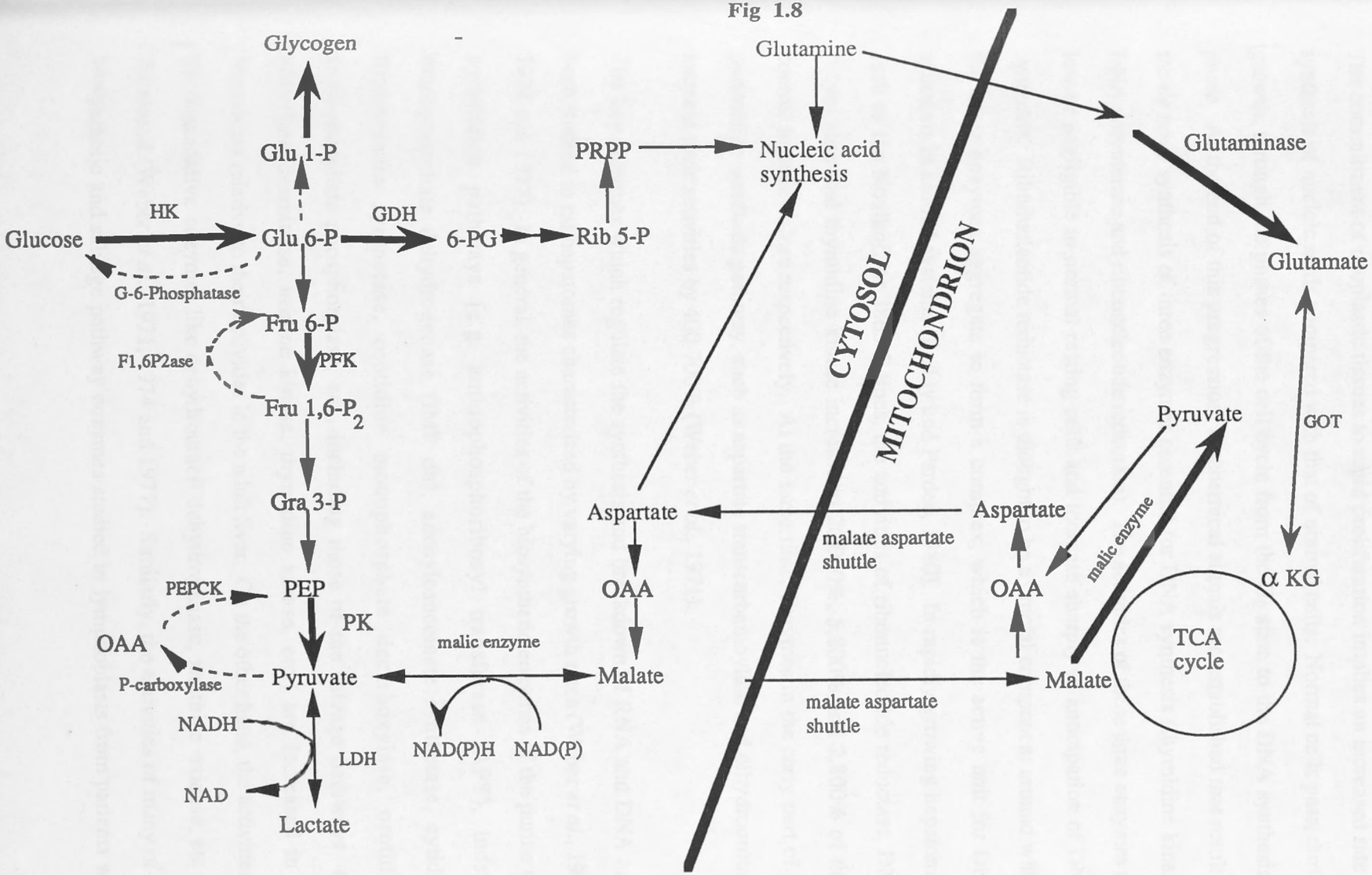
The rate of lipid synthesis varies considerably in hepatomas and may be more than, less than, or the same as, that in normal liver. Sabine *et al.* (1968) observed no relationship between the rate of tumour growth and rate of fatty acid synthesis *in vitro*. However, Sabine (1976) and Coleman and Lavietes (1981) have demonstrated greatly enhanced and unregulated cholesterogenesis in tumour cells. Loss of feed-back control of cholesterol and/or fatty acid synthesis has been demonstrated in hepatomas, pre-neoplastic liver and leukaemias (Goldforb and Pitot, 1971; Sabine 1975 and 1976; Halpercin *et al.*, 1975; Chen *et al.*, 1978).

Increased rates of lipid synthesis in hepatomas are significant in relation to the flux of glucose through the oxidative segment of the pentose phosphate pathway, since this flux is accompanied by the production of NADPH as an essential source of reducing equivalents for the biosynthesis of lipids. It has been shown in cultured He La cells that the cytoplasmic malic enzyme is a more important source of NADPH than synthesis via the oxidative segment of the pentose pathway (Reitzer *et al.*, 1980). The role of the mitochondrial $NADP^+$-linked malic enzyme might be viewed, in this light, as an essential link in the oxidative conversion of malate to citrate. Citrate extruded from mitochondria is the primary cytosolic precursor of acetyl CoA for cholesterol and fatty acid biosynthesis. Hence mitochondrial malic enzyme, which is present exclusively in rapidly-proliferating cells and tumour cells, would provide pyruvate derived, ultimately, from glutamine for subsequent conversion to citrate. Cells with these characteristics would exhibit enhanced and unregulated cholesterogenesis (Moreadith and Lehninger, 1984).

Fig 1.8:- Glycolysis and glutaminolysis in neoplastic cells.

Dark arrows indicate enzymes activated, dotted lines denote enzymes inhibited and normal arrows show enzymes which have the same level of activity as in the normal cells. Abbreviation used: Glu 1-P, Glucose 1-phosphate; Glu 6-P, Glucose 6-phosphate; Fru 6-P, Fructose 6-phosphate; Fru 1,6-P_2, Fructose 1,6-diphosphate; Gra 3-P, glyceraldehyde 3-phosphate; PEP, phosphoenol pyruvate; OAA, oxaloacetate; Rib 5-P, ribose 5-phosphate; PRPP, 1-pyrophosphorylribosyl-5-phosphate; α-KG, α-ketoglutarate; HK, hexokinase; PEPCK, phosphoenol pyruvate carboxykinase; PK, pyruvate kinase; LDH, lactate dehydrogenase; P-carboxylase; pyruvate carboxylase; GOT, glutamate-oxaloacetate transaminase; GDH, glucose 6-phosphate dehydrogenase; 6-PG, 6 phosphogluconate.

Fig 1.8


Glycogen
Glu 1-P
HK
Glucose
Glu 6-P
GDH
6-PG
Rib 5-P
PRPP
Nucleic acid synthesis
Glutamine
Glutaminase
Glutamate
G-6-Phosphatase
Fru 6-P
F1,6P2ase
PFK
Fru 1,6-P2
Gra 3-P
PEPCK
PEP
PK
OAA
P-carboxylase
Pyruvate
NADH
LDH
NAD
Lactate
malic enzyme
NAD(P)H
NAD(P)
Malate
CYTOSOL
MITOCHONDRION
Aspartate
OAA
malate aspartate shuttle
Aspartate
OAA
malic enzyme
Pyruvate
GOT
α KG
TCA cycle
Malate
malate aspartate shuttle

1.6.4 Nucleic acid metabolism

The commitment of neoplastic tissues to rapid proliferation implies an increased rate of synthesis of nucleic acids compared with that of normal cells. Normal cells pass, during growth, through the phases of the cell cycle from the G_0 state to the DNA synthesis S phase. At the end of this progression, biochemical signals are established that result in the *de novo* synthesis of three enzymes essential for DNA synthesis (thymidine kinase, DNA polymerase and ribonucleotide reductase). The activities of these three enzymes are low or negligible in normal resting cells and increase sharply in anticipation of DNA synthesis. Ribonucleotide reductase is thought to be a crucial component around which the other enzymes aggregate to form a complex, which is the active unit for DNA synthesis in animal systems (Reddy and Pardee, 1980). In rapidly-growing hepatomas, such as the Novikoff or 3863-F lines, the activities of ribonucleotide reductase, DNA polymerase and thymidine kinase increase to 20,000%, 5,800% and 2,800% of their control hepatic values respectively. At the same time, enzymes in the early part of the pyrimidine synthesis pathway, such as aspartate transcarbamoylase and dihydroorotase, increase their activities by 400-700% (Weber *et al.*, 1971).

The key enzymes which regulate the synthesis and breakdown of RNA and DNA have been studied in rat hepatomas characterized by varying growth rates (Weber *et al.*, 1971, 1974 and 1977). In general, the activities of the biosynthetic enzymes of the purine and pyrimidine pathways [e.g. amidophosphoribosyl transferase (APT), inosine monophosphate dehydrogenase (IMP dh), adenylosuccinate synthetase, cytidine triphosphate synthetase, orotidine monophosphate decarboxylase, orotidine monophosphate phosphorylase, etc., including those of the salvage pathways e.g. adenylate deaminase, uridine kinase, thymidine kinase, etc.] are increased in all hepatomas relative to their activities in the adult liver. On the other hand, the activities of the degradative enzymes like dehydrouracil dehydrogenase, xanthine oxidase, etc are decreased (Weber *et al.*, 1971, 1974 and 1977). Similarly, the activities of many of the biosynthetic and salvage pathway enzymes studied in lymphoblasts from patients with

acute lymphocytic leukaemia are several-fold higher than the activities of the corresponding enzymes in normal lymphocytes (Scholar and Calabresi, 1973). A related study involving murine leukaemic cell lines L5778Y and L1210 and murine ascites sarcoma 180 cells has shown similar enzymatic patterns, but with excessively elevated activities compared with those found in lymphocytes from normal donors (Scholar and Calabresi, 1973). An integrated pattern of metabolic pathways is shown in Fig 1.9.

The results indicate that regulation of nucleic acid synthesis is coordinated by reciprocal regulation of the synthesis of enzymes which catalyze the steps of the biosynthetic and degradative pathways. The same phenomenon has been observed in the regulation of ornithine metabolism in hepatomas with varying growth rates (Williams-Ashman *et al.*, 1972; Weber *et al.*, 1971). An increase in the synthesis of polyamines (spermidine and spermine), preceded by increased activity of ornithine decarboxylase, occurs in the hepatomas in the face of a pronounced decrease in ornithine carbamyl transferase activity, resulting in a decline in the activity of the urea cycle. A summary of the regulation of the 'opposing' metabolic pathways is presented in Table 1.1.

1.7 Control of metabolism in neoplasia or/differentiation

It was first established in the early 1960's that the neoplastic or differentiated state of the cell is implemented by controls operating at the genomic level (Gourdan, 1962). It was also shown that the particular phenotype of a cell is not permanent but can be altered under appropriate conditions. Changes in gene expression during neoplasia or/differentiation are reflected in the changes in mRNA and protein content of the cell. This has been reported frequently in studies of the induced transformation or/differentiation of various cell lines (Weber *et al.*, 1974; Colbert *et al.*, 1983; Reyland *et al.*, 1986).

There are many stages on the pathway from gene to protein at which control mechanisms could operate to regulate the final functional protein profile of the cell and hence determine the cell's ultimate neoplastic or/differentiated phenotype. The molecular

Fig 1.9:- Integrated pattern of glycolysis, gluconeogenesis, pentose phosphate pathway, purine and pyrimidine synthesis and degradation in neoplasia.

Dark arrows indicate enzymes activated, dotted lines denote enzymes inhibited and normal arrows show enzymes which are the same as in the normal cells. Abbreviation used: Glu 1-P, Glucose 1-phosphate; Glu 6-P, Glucose 6-phosphate; Fru 6-P, Fructose 6-phosphate; Fru 1,6-P_2, Fructose 1,6-diphosphate; Gra 3-P, glyceraldehyde 3-phosphate; PEP, phosphoenol pyruvate; OAA, oxaloacetate; Rib 5-P, ribose 5-phosphate; PRPP, 1pyrophosphorylribosyl-5-phosphate; α-KG, α-ketoglutarate; HK, hexokinase; PEPCK, phosphoenol pyruvate carboxykinase; PK, pyruvate kinase; LDH, lactate dehydrogenase; P-carboxylase, pyruvate carboxylase; GOT, glutamate-oxaloacetate transaminase; GDH, glucose 6-phosphate dehydrogenase; RD, ribonucleotide reductase; 6-PG, 6 phosphogluconate; Seh 7-P, sedoheptulose 7-phosphate; Ru 5-P, ribulose 5-phosphate; 5-PRA, 5' phosphoribosylamine; IMP, inosine monophosphate; OMP, oritidine monophosphate; GMP, guanosine monophosphate; AMP, adenosine monophosphate; UMP, uridine monophosphate; CMP, cytidine monophosphate; TMP, thymidine monophosphate; GDP, ADP, UDP, CDP, TDP, are the respective diphosphates; GTP, ATP, UTP, CTP, TTP, are the respective triphosphates; d-nucleotides are the deoxy nucleotides.

Fig 1.9

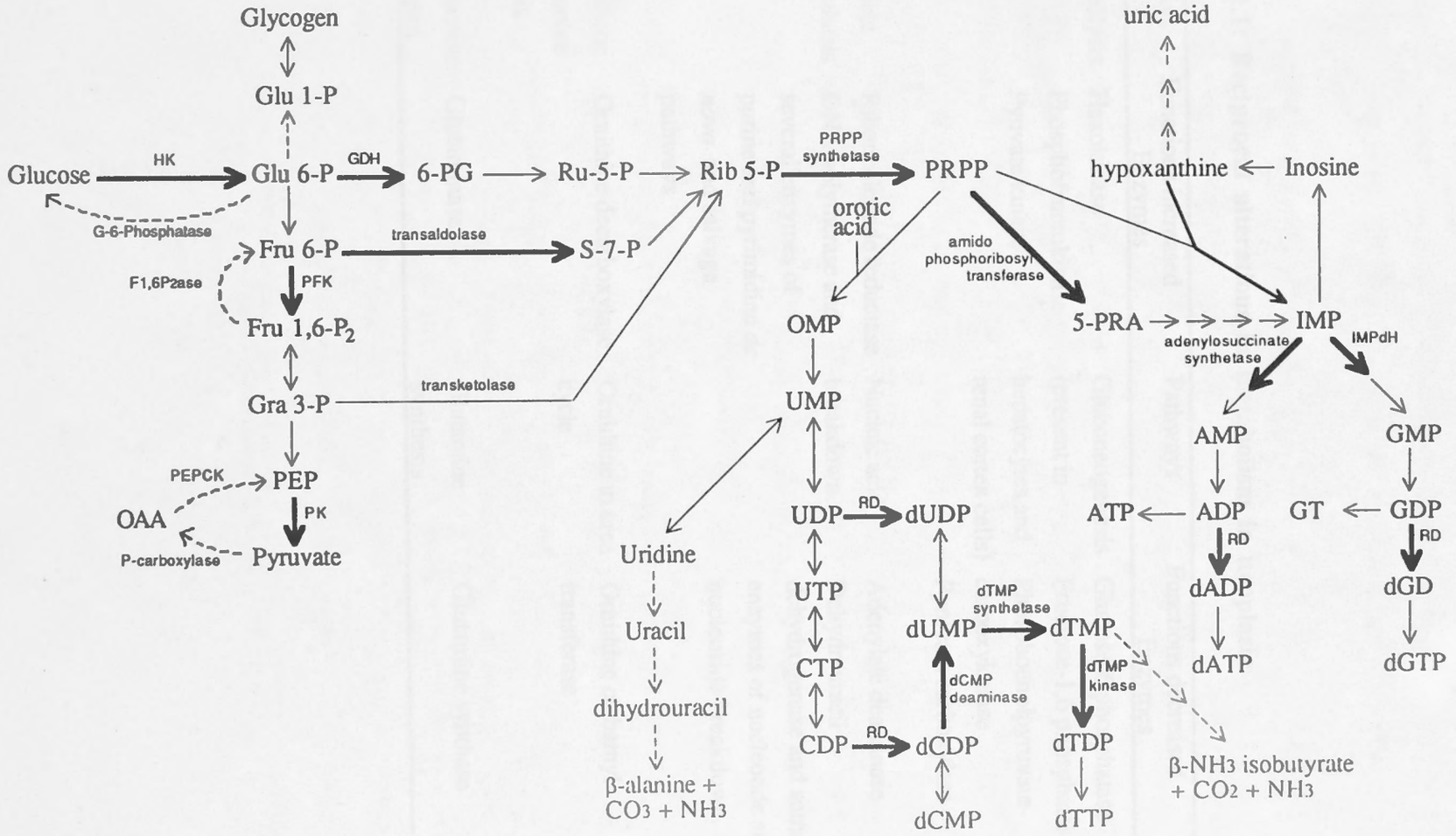

Glycogen
Glu 1-P
Glucose
HK
Glu 6-P
GDH
6-PG
Ru-5-P
Rib 5-P
PRPP synthetase
PRPP
G-6-Phosphatase
Fru 6-P
transaldolase
S-7-P
F1,6P2ase
PFK
Fru 1,6-P2
Gra 3-P
transketolase
PEPCK
PEP
OAA
PK
P-carboxylase
Pyruvate
uric acid
hypoxanthine
Inosine
orotic acid
amido phosphoribosyl transferase
OMP
5-PRA
IMP
adenylosuccinate synthetase
IMPdH
UMP
AMP
GMP
UDP
RD
dUDP
ATP
ADP
GT
GDP
RD
RD
Uridine
UTP
dTMP synthetase
dUMP
dTMP
dADP
dGD
Uracil
CTP
dCMP deaminase
dTMP kinase
dATP
dGTP
dihydrouracil
CDP
RD
dCDP
dTDP
β-NH3 isobutyrate + CO2 + NH3
β-alanine + CO3 + NH3
dCMP
dTTP

Table 1.1: Reciprocal alterations in metabolism in neoplasia

Pathways	Functions increased Enzymes	Pathways	Functions decreased Enzymes
(1) Glycolysis	Hexokinase Phosphofructokinase Pyuvate kinase	Gluconeogenesis (present in hepatocytes and renal cortex cells)	Glucose-6 phosphatase Fructose-1,6 phosphatase Phosphoenolpyruvate-carboxykinase Pyruvate carboxylase
(2) Nucleic acid synthesis	Ribonucleotide reductase DNA polymerase and several enzymes of purine and pyrimidine *de novo* and salvage pathways	Nucleic acid breakdown	Adenylate deaminase Dehydrouracil dehydrogenase and some enzymes of nucleotide and nucleoside breakdown.
(3) Ornithine to polyamine synthesis	Ornithine decarboxylase	Ornithine to urea cycle	Ornithine carbamyl transferase
(4) Glutamine breakdown	Glutaminase	Glutamine synthesis	Glutamine synthase

mechanisms which regulate the expression of genes coding for single proteins or a group of functionally-related enzymes to bring about the changes in various metabolic pathways associated with neoplasia/or differentiation may act at the following cellular levels:

1.7.1 Control at the genetic level

At the genetic level, alterations in chromatin structure may affect gene transcription. Active chromatin, believed to occur at sites of frequent transcriptional activity, appears to have a more 'open' structure than that of condensed inactive chromatin (Weisbrod, 1982). Moreover, chemical modification (such as acetylation, methylation, phosphorylation, etc) of proteins associated with active chromatin can also lead to altered gene expression. Changes in histone association with DNA have been observed during chick and murine development and during Friend erythroleukaemic cell differentiation (Newrock *et al.*, 1976; Cohen *et al.*, 1979; Grove and Zweilder, 1984). Moreover, methylation and acetylation of DNA also cause changes at the site of active gene transcription that may lead to stimulated transcription.

At the genetic level, control can be exerted at the structural or regulatory gene levels. The structural genes that encode the key regulatory enzymes may be present or absent (due to chromosomal deletion). If they are present, they may be active or inactive, leading to the possibility of an all-or-none type of expression. The active gene may undergo alteration, resulting in the expression of an isoenzyme pattern characteristic of the neoplastic or/differentiated phenotype. The regulatory genes may act as integrator genes which control the activities of groups of functionally-linked enzymes: for example, the enhanced expression of the key enzymes of the glycolytic pathway, the pentose phosphate pathway and nucleic acid synthesis in neoplasia.

1.7.2 Control at the cytoplasmic and translational level

After passing into the cytoplasm, mRNA carrying functionally-coded recognition sites may be preserved or selectively destroyed by specific enzymes, permitting the selective translation of groups of mRNA associated with a particular phenotype, whilst denying the translation of those involved in the opposing pathway (Weber, 1983).

1.7.3 Control at the post-translational level

Following translation, a protein may require a number of modifications to become fully functional. These include the addition of prosthetic groups, glycosylation, methylation or phosphorylation. Proteolysis of the primary translational product may also be required. Regulation may also be exerted at the enzymic level before or after the enzyme has achieved its activity by appropriate folding. The enzymes of antagonistic pathways may be destroyed by function-specific proteases that do not affect the activities of the induced enzymes.

Changes in protein glycosylation of the primary translation product that occur during differentiation have been discussed in Section 1.5.4. The addition of prosthetic groups has also been recognized as occupying a prominent position in the pathways that govern the phenotype of cells. Phosphorylation of specific amino acid residues is crucial for the function of many proteins (Hunter and Cooper, 1985). Moreover some oncogenes (e.g. retroviral oncogene *v-src* and its cellular progenitor *c-src*) are tyrosine-specific protein kinases that act as transmembrane receptors with the capacity to initiate a chain of events specific for either differentiation or proliferation.

Hence, the regulation of metabolism associated with neoplasia or/differentiation may be controlled at different cellular levels, resulting in the expression of a biochemical phenotype that represents the emergence of integrated reprogramming of gene expression.

1.8 Phenotypic expression associated with differentiation of neoplastic cells with particular reference to leukaemic cells

Terminal differentiation is associated with a blockade of proliferation and inhibition of DNA synthesis. The first phenotypic expression of terminal differentiation is partial or complete inhibition of the key regulatory enzymes of DNA synthesis (e.g. thymidine kinase, DNA polymerase and ribonucleotide reductase). Besides these, the enzymes of purine and pyrimidine biosynthesis are also inhibited. In murine virus-induced erythroleukaemic cells differentiated with DMSO, amidophosphoribosyl transferase (the first key regulatory enzyme of the *de novo* purine synthesis pathway) is inhibited, resulting in the total inhibition of *de novo* purine synthesis in the differentiated cells (Reem and Friend, 1975). Anti-metabolites which inhibit the enzymes of purine and pyrimidine pathways differentiate leukaemic cells by inhibiting these two essential pathways (Knight *et al.*, 1987; Nichols *et al.*, 1989; and Makishima *et al.*, 1991). These findings suggest that the most fundamental expression of the differentiated phenotype is to exhibit no (or only nominal) proliferation by inhibition of DNA synthesis at the genetic/transcriptional/translational/or post-translational levels.

Most leukaemic cells are immature blast cells which enter the circulation and proliferate at different stages of maturation. These cells have multi-potential capabilities and can be differentiated into different variety of lineages under the influence of differentiating signals. Depending on their state of maturation and the differentiating agents used, these cells can be differentiated to erythrocytes, granulocytes, monocytes and macrophages. Recent studies with HL60 cells have shown that they also retain the potential of being differentiated to osteoclasts, the unique multinucleated cells responsible for bone degradation under physiological and pathophysiological conditions (Yoneda *et al.*, 1991). The ultimate phenotype depends on the type of differentiation the leukaemic cells have undergone, e.g. Friend erythroleukaemic cells (Reem and Friend, 1975) and human K562 cells (Marie *et al.*, 1981) exhibit haemoglobin synthesis when differentiated to erythrocytes while the parental leukaemic cells lack this capability. Cells (HL60, U937,

etc) which differentiate to granulocytes, monocytes and macrophages, collectively known as phagocytes, display differentiated microbicidal activity consisting of a combination of oxidative and enzymatic (non-oxidative) processes. The oxidative mechanism known as the 'respiratory (or oxidative) burst' is composed of an NADPH-oxidase which consists of a transmembrane electron transport chain that utilizes cytosolic NADPH as the donor and oxygen as the acceptor of single electrons leading to the formation and release of reactive oxygen intermediates (ROI) into the extracellular space or into phagocytic vacuoles (Baggiolini and Wymann, 1990). Associated with the oxidative burst is degranulation, which results in the release of myeloperoxidase (MPO) from the azurophilic (primary) granules and hydrolytic enzymes from both the secondary and azurophilic granules (Klebanoff *et al.*, 1986).

1.8.1 Oxidative processes

a) Formation of reactive oxygen intermediates

The 'oxidative or respiratory burst' is characterized by a large uptake of oxygen (that is non-mitochondrial and insensitive to cyanide inhibition) by the phagocytes, accompanied by increased metabolism of [1-^{14}C] glucose via the oxidative segment of the pentose phosphate pathway (Fantone and Ward, 1982). The burst is triggered when phagocytes make contact with 'foreign targets' via complementary receptors on the external surface of the phagocyte membrane. The burst can also be triggered by soluble stimuli (e.g. PMA) that activate reactive oxygen intermediate formation independently of phagocytosis. The activation of the oxidative burst has been studied most thoroughly in neutrophils stimulated with chemotactic receptor agonists (Baggiolini and Wymann, 1990). It is generally accepted that activation is initiated by the formation of a receptor-ligand complex which results in the activation of a GTP-binding protein(s). Dissociation of the GTP-bound α subunit from the heterotrimeric G protein induces the activation of phospholipase C, which in turn cleaves phosphatidyl inositol 4,5-bisphosphate into IP_3 and DAG. The rise in intracellular calcium caused by elevated IP_3 levels, leading to protein phosphorylation catalyzed by PKC, is thought to be the major route responsible

for the activation of the NADPH oxidase (Rossi, 1986). The receptor may also stimulate, through a G-protein, an alternative route involving a Ca^{2+}/ calmodulin protein kinase and/ or a tyrosine-specific protein kinase, which has been shown recently to be activated by chemoattractants (Huang *et al.*, 1988). The existence of a novel GTP-binding protein which lies physically close to the NADPH oxidase, and is capable of modulating its activity, has been reported recently (Lu and Grienstein, 1990). The activation of oxidative burst activity and the resulting formation of reactive oxygen intermediates (ROI) and degranulation is shown in Fig 1.10 (a and b).

The oxidative burst results in the sequential production of a variety of ROI (Fig 1.10 c). Superoxide ($O_2^{-}\cdot$) is formed, initially, by the reduction of molecular oxygen by single electrons derived from NADPH generated via the pentose phosphate pathway. Superoxide production is catalyzed by the combined action of a plasma membrane NADPH oxidase and cytochrome b_{558} which is the terminal electron acceptor of a short electron transport chain that conveys electrons from NADPH to oxygen (Baboir, 1987; Segal, 1989). Subsequent ROI formed from superoxide include: (1) hydrogen peroxide (H_2O_2), formed either by spontaneous dismutation or by the catalytic action of superoxide dismutase (SOD); (2) MPO-dependent oxyhalide formation (e.g. HOCl); (3) hydroxyl radical formation (OH·); and (4) the formation of singlet oxygen ($^1O_2$). Of all the ROIs produced, the hydroxy radical is the most toxic known. Its formation involves the reduction of H_2O_2 by Fe^{+2} derived from Fe^{+3}, which is stored in abundance in lactoferrin present in phagocytes (Fantone and Ward, 1982). The exact mechanism by which each of these ROI contribute to microbicidal killing is not known (McKenna and Davies, 1988). Superoxide sustains MPO activity and HOCl production, while SOD is inhibitory because supersaturating levels of H_2O_2 promote the formation of an inactive form of MPO (Compound 11) (Kettle and Winterbourn, 1988).

The mechanisms by which the oxidative burst is terminated are not known, but inactivation may occur when activation factors are consumed or when the oxidase is inactivated by its own products (oxidants) or by hydrolytic enzymes. Termination may

Fig 1.10 (a):- Schematic diagram of the possible sites and mechanisms of activation of the oxidative burst.

R, receptor; G, heterotrimeric GTP-binding protein; and G_N, newly discovered GTP-binding protein. Solid arrows indicate established stimulatory reactions, dotted lines, speculative stimulatory reactions. The receptor, through GTP-binding protein, activates two separate ATP-dependent pathways: (1) involving PLC and PKC; (2) stimulated by Ca^{2+}/ calmodulin dependent kinase or tyrosine kinase at a site involving G_N, after the ATP-requiring step; (3) is the ATP-independent pathway involving only G_N. The mechanism of activation of the oxidative burst presented is essentially the same as that suggested by Lu and Grinstein (1990).

Fig 1.10 (b):- The reactions of the oxidative burst, as they occur at the inner membrane of the phagolysosome.

Fig 1.10 (a)

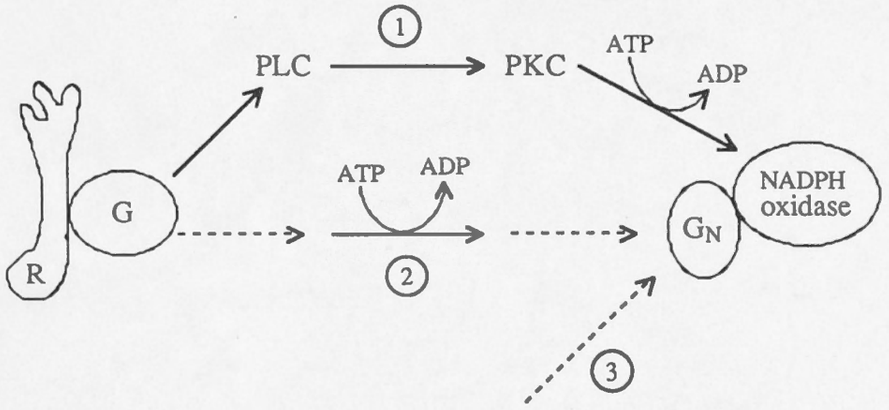


Fig 1.10 (b)

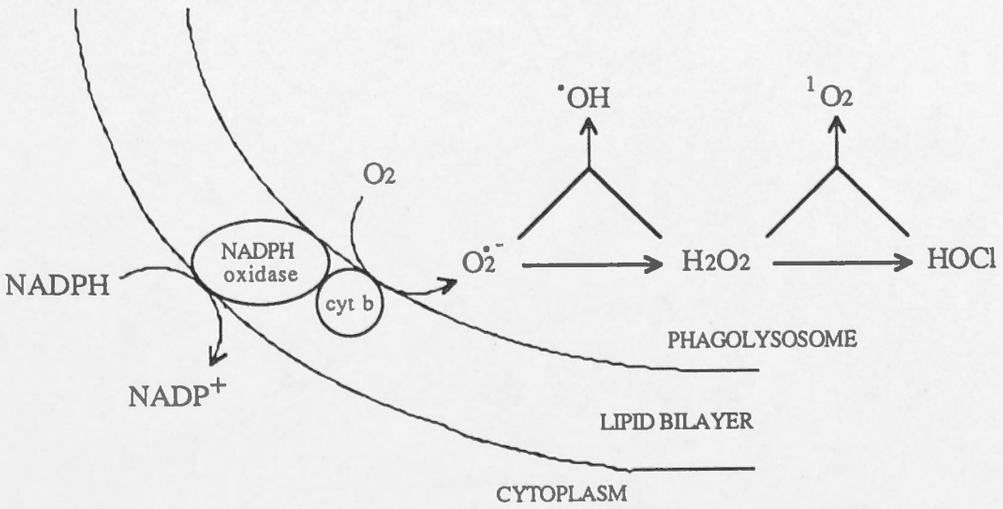


Fig 1.10 (c):- The reactions of the oxidative burst.

The first three reactions are established reactions, while the other reactions may occur at low rates. SOD is superoxide dismutase and MPO is myeloperoxidase.

Fig 1.10 (c)

$$2O_2 + NADPH \xrightarrow{\text{NADPH oxidase}} 2O_2^{\bullet -} + NADP^+ + H^+$$

$$2O_2^{\bullet -} + 2H^+ \xrightarrow[\text{and SOD}]{\text{spontaneous}} H_2O_2 + O_2$$

$$H_2O_2 + Cl^- + H^+ \xrightarrow{\text{MPO}} HOCl + H_2O$$

and possibly

$$O_2^{\bullet -} + H_2O_2 \xrightarrow{\text{iron ligands}} {}^{\bullet}OH + OH^- + O_2$$

$$H_2O_2 + HOCl \xrightarrow{\text{spontaneous}} {}^1O_2 + Cl^- + H^+ + H_2O$$

also proceed by specific processes such as the dephosphorylation or phosphorylation of phosphorylated or unphosphorylated components or by 'feed-back' control or 'down-regulation' of the receptors that maintain steady-state levels of PKC activators (DAG, Ca^{+2}, IP_3, etc). PKC itself has been proposed to participate in the 'down-regulation' of some receptors (Nishizuka, 1986).

b) Formation of reactive nitrogen intermediates

Whilst the importance of the oxidative burst is well established as a cytotoxic mechanism, less is known about the production of reactive nitrogen intermediates (RNI) by phagocytes. RNI may contribute to microbicidal activity by reacting with ROI to form secondary cytotoxic species like peroxynitrite (Beckman *et al.*, 1990). The major *physiological* role of these species appears to be in the regulation of vascular tone by activating guanylate cyclase in smooth muscle cells (Moncada *et al.*, 1990). RNI may also contribute to the tumouricidal activity of activated macrophages (Hibbs *et al.*, 1987). In mammary tumour cells and in some adenocarcinoma cells, NO· inhibits DNA synthesis by inhibiting ribonucleotide reductase (Lepoivre *et al.*, 1990).

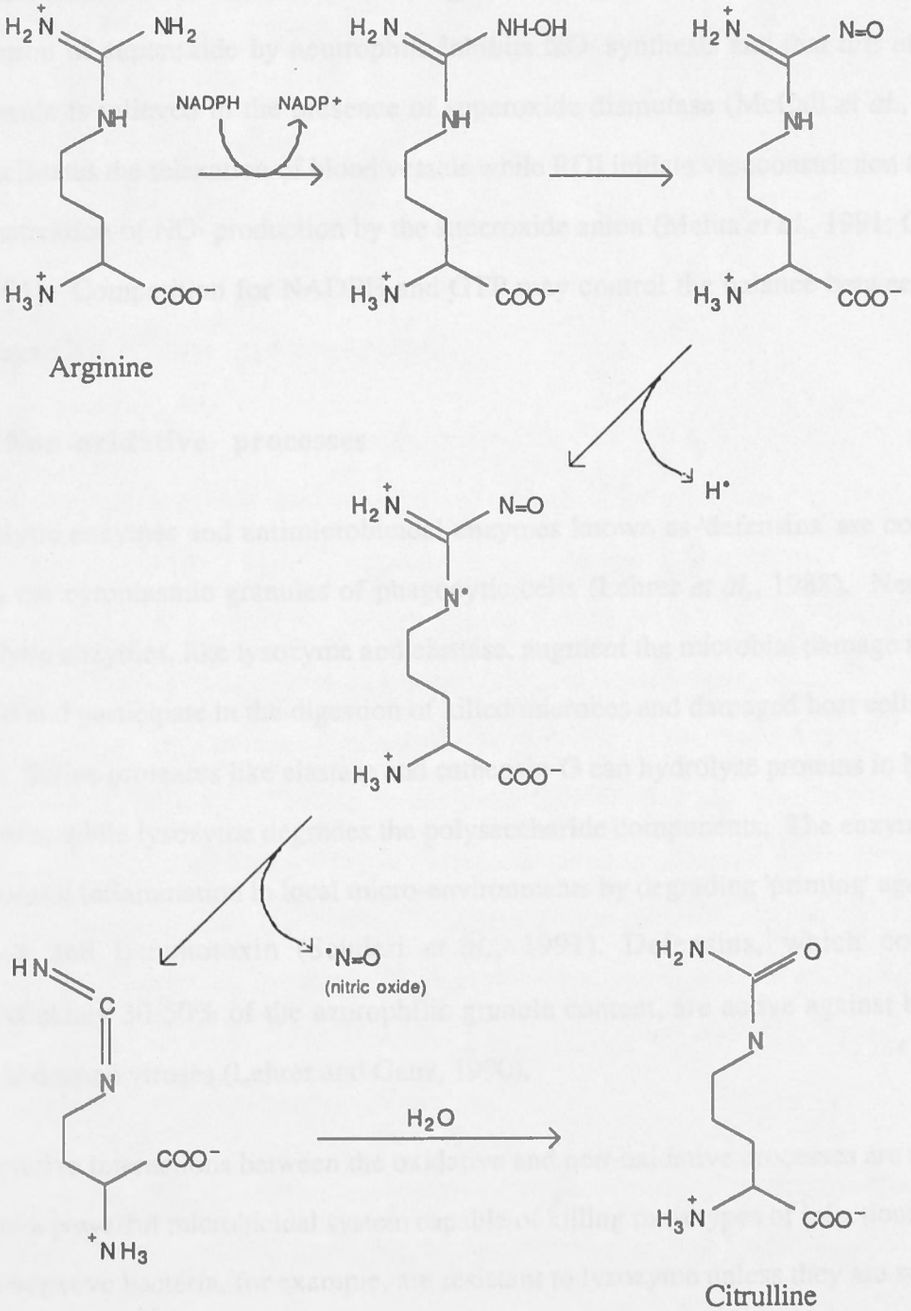
The exact mechanism of production of NO· is not known with certainty, but it is thought to be derived from the guanido nitrogen of L-arginine by a reaction catalyzed by nitric oxide synthase, an enzyme present in many types of cells (e.g. brain, muscle, platelets, neutrophils, macrophages, etc.). The most likely mechanism of NO· production, as proposed by Marletta *et al* . (1988), is given in Fig 1.11. NO· is very unstable in tissues and it is converted spontaneously to NO_2^- and NO_3^- (Marletta *et al.*, 1988). NO· is produced by two types of nitric oxide synthase: (1) a constitutive type responsible for the release of NO· for physiological transduction purposes; while the other (2) is induced by cytokines and miscellaneous agents (e.g. DMSO) that stimulate release of NO· as part of the immunological response (Moncada and Higgs, 1991).

ROI and RNI are produced by independent pathways and, thus, the oxidative burst is not involved directly in RNI synthesis (Ding *et al.*, 1988). NO· is produced continuously in

Fig 1.11:- Possible mechanism of formation of $NO^{\cdot}$ from L-arginine.

The first step is a monooxygenase-type reaction occuring at one of the guanido nitrogens, generating N^{G}-hydroxyl-L-arginine. The reaction requires reducing equivalents and is the likely step for the involvement of NADPH. The next step is a two-electron oxidation to a nitrosoamidine-like intermediate, followed by homolytic cleavage of the C-NO bond, generating $NO^{\cdot}$ and the diimide of ornithine. Reaction of this diimide with water yields citrulline, the other end product.

Fig 1.11



unstimulated neutrophils, but stimulation by a chemoattractant agonist stops this pathway in favour of the oxidative burst (Wright *et al.*, 1989). Thus, the two pathways may compete for common intermediates (e.g. NADPH). It has been shown that the production of superoxide by neutrophils inhibits NO· synthesis and that this effect of superoxide is relieved in the presence of superoxide dismutase (McCall *et al.*, 1989). NO· facilitates the relaxation of blood vessels while ROI initiate vasoconstriction through the inactivation of NO· production by the superoxide anion (Mehta *et al.*, 1991; Csaki *et al.*, 1991). Competition for NADPH and GTP may control the balance between these pathways.

1.8.2 Non-oxidative processes

Hydrolytic enzymes and antimicrobicidal enzymes known as 'defensins' are contained within the cytoplasmic granules of phagocytic cells (Lehrer *et al.*, 1988). Neutrophil hydrolytic enzymes, like lysozyme and elastase, augment the microbial damage initiated by ROI and participate in the digestion of killed microbes and damaged host cells (Roitt, 1984). Serine proteases like elastase and cathepsin-G can hydrolyse proteins in bacterial cell walls, while lysozyme degrades the polysaccharide components. The enzymes may also control inflammation in local micro-environments by degrading 'priming' agents like TNF-α and lymphotoxin (Scuderi *et al.*, 1991). Defensins, which constitute approximately 30-50% of the azurophilic granule content, are active against bacteria, fungi and some viruses (Lehrer and Ganz, 1990).

Cooperative interactions between the oxidative and non-oxidative processes are required to form a powerful microbicidal system capable of killing most types of infectious agents. Gram negative bacteria, for example, are resistant to lysozyme unless they are subjected simultaneously to ROI (Lehrer and Ganz, 1990). Defensins and ROI interact synergistically to lyse tumour cells *in vitro* (Lichenstein *et al.*, 1988).

1.9 The human promyelocytic leukaemic HL60 cell line

The HL60 cell line, derived from a 35 year old female patient with acute promyelocytic leukaemia, was isolated some 15 years ago in the laboratory of R. C. Gallo at the National Institutes of Health (NIH) in an attempt to identify some leukaemia-specific growth factors that sustain leukaemic growth *in vivo*. This cell line provides a unique model system for studying *in vitro* the cellular and molecular events involved in the proliferation and differentiation of normal and leukaemic cells of the granulocyte/monocyte/macrophage lineages.

The HL60 cell line has a population doubling time of 20-24 hours. In culture the great majority of these cells stain as myeloblasts and promyelocytes, although 5-10% appear to have passed this stage. HL60 is a constitutive producer of a glycoprotein growth factor upon which it is dependent for colony growth (Perkins *et al.*, 1984). Functional and biochemical studies indicate that this autostimulatory activity is distinct from CSF. This type of autocrine growth regulation is probably important in the maintenance of neoplasia (Harris and Ralph, 1985). These cells can proliferate in serum-free media provided they are supplemented with transferrin and insulin (Breitman *et al.*, 1980). The requirement for insulin and transferrin is absolute. Receptors for these ligands are displayed by HL60 cells, but this does not explain the unusual proliferative capacity of this cell line, as a number of other leukaemic cells that display the same receptors are unable to grow continuously in culture (Collins, 1987).

Histochemically, HL60 cells have a positive profile for myeloperoxidase, chloroacetate esterase, acid phosphatase, lysozyme, beta-glucuronidase and Sudan Black B. These cells have marginal capacity to produce ROI and have low levels of enzymes of the oxidative segment of the pentose phosphate pathway (Newburger *et al.*, 1979). They grow as compact colonies in semisolid culture, do not adhere to the substrata, possess receptors for Fc-IgG_1, C_3b and the tripeptide formyl-methionyl-leucyl-phenylalanine and are weakly phagocytic for latex beads or yeast particles (Harris and Ralph, 1985).

Cytogenetic analyses of HL60 cells reveals many karyotypic abnormalities, including the deletion of the p53 gene on chromosome 17 (Wolf and Rotter, 1985), and deletion of the genes for GM-CSF, G-CSF, IL-3, IL-4, IL-5 and *c-fms* on chromosome 5 (LeBeau *et al.*, 1989). The most important of all these alterations is the several-fold amplification of DNA sequences encompassing the *c-myc* gene (Collins and Groudine, 1982). HL60 cells form tumours in 50% of the mice injected (Potter *et al.*, 1985). The tumours consist primarily of promyelocytes and myeloblasts with no detectable metastases. HL60Nu1 cells established from one of these tumours are the most tumourigenic, with 100% of the injected mice developing tumours containing as many as 10^7 cells (Gallagher *et al.*, 1979).

1.9.1 Induced differentiation of HL60 cells

Various agents induce HL60 cells to differentiate to four general types of non-proliferating cells: (a) granulocytes, (b) monocytes, (c) macrophages and (d) eosinophils. Certain agents give rise to cells with characteristics that overlap these categories. The phenotypic characteristics of induced and uninduced HL60 cells are given in Table 1.2.

(a) Granulocytes: Inducing agents such as DMSO with polar-planar characteristics and some other agents (such as RA), differentiate HL60 cells to cells with morphological, functional, enzymatic and surface membrane antigen properties characteristic of mature granulocytes (Table 1.2). By several criteria this granulocytic differentiation of HL60 cells is incomplete and defective. For example, most granulocyte-induced cultures contain predominantly metamyelocytes and banded neutrophils rather than fully-differentiated multilobulated polymorphs. In addition, the granulocyte-induced HL60 cells lack lactoferrin, suggesting that they are deficient in secondary granules (Newburger *et al.*, 1979). The lactate dehydrogenase (LDH) isoenzyme profile of granulocyte-induced HL60 cells also differs quantitatively from the LDH isoenzymes of normal granulocytes, consistent with incomplete differentiation (Pantazis *et al.*, 1981). Deficiencies have also been described for the MPO system (Pullen and Hosking, 1985). Despite these deficiencies, most of the granulocyte-inducing agents induce terminal

differentiation of HL60 cells, so that the successfully induced cells no longer retain their proliferative capacity.

(b) Monocytes: Many compounds are capable of inducing monocytic differentiation of HL60 cells. Of these, butyrate and vitamin D_3 (Calcitriol) are the best known (Boyd and Metcalf, 1984; Tanaka *et al.*, 1982). Monocytic differentiated HL60 cells can be confused with immature HL60 granulocytes, and they also share many important functional characteristics with HL60 cells treated with granulocytic inducers (Table 1.2). They can be readily distinguished from these latter cells, however, by specific markers, including positive staining for the monocyte/macrophage specific enzyme α-napthyl acetate esterase and a number of monocyte-specific cell membrane antigens (Todd *et al.*, 1981).

Many naturally-occurring compounds (cytokines) are also capable, in synergism, of inducing monocytic differentiation in HL60 cells. Among these, DIF, IFN-γ and IFN-α in combination with RA induce monocytic differentiation in HL60 cells (Olsson *et al.*, 1982; Hemmi and Breitman, 1987; Grant *et al.*, 1985). Monocytic differentiation of HL60 cells, unlike granulocytic differentiation, is not necessarily accompanied by the inhibition of proliferation. For example, the combination of RA+interferon-γ (γ-IFN) induce monocytic differentiation in HL60 cells without the loss of proliferative potential (Hemmi and Breitman, 1987).

(c) Macrophage-like: The best example of macrophage-like induced HL60 cells is that induced by the tumour promoter PMA. These cells share many of the same enzymatic, histochemical and functional characteristics of the monocytic-induced HL60 cells. The phenotypic characteristic which distinguishes them clearly from the monocytic-differentiated cells is their intense adherence to plastic, with formation of prominent pseudopodia (Rovera *et al.*, 1979). Several specific surface antigens present on normal and monocytic-induced HL60 cells are not induced in PMA-treated HL60 cells (Ferrero *et al.*, 1983). For example, there is no induction of chemotactic peptide receptors and ROI, whereas these are the significant characteristics of HL60 monocytes (Newburger *et al.*,

Table 1.2 : Phenotypic characteristics of uninduced and induced HL60 cells.

Characteristics	Uninduced HL60	Granulocytes	Monocytes	Macrophage -like	Eosinophils
Myeloperoxidase	+	-	--	--	+
Specific esterase	+	+	±	-	+
Non-specific esterase	+	-	+	+	-
Acid phosphatase	+	++	++	+++	+
Plastic adherence	-	-	-	+++	-
Chemotaxis	-	+	+	-	
Chemotactic receptors	+	++	++	++	
Complement- receptors	+	++	++	++	
Fc receptors	+	++	++	++	
Lysozyme	+	++	++	+++	
NBT reduction	+	+++	++	-	
Phagocytosis	-	++	++	++	
Insulin receptors	+	--	--	--	
Transferrin receptors	+	--	--	--	
Granulocyte surface antigens	±	+	-	-	
Monocyte/macrophage surface antigens	±	±	+	+	
Production of ROI	+	+++	++	-	

Blank space correspond to characteristics absent from the literature. (+) indicates present, (-) indicates absent, (++) indicates increase, (--) indicates decrease, (±) indicates may or may not be present and (+++) indicates increases more than (++).

1982). Like granulocytic induction, macrophage-like induction of HL60 cells by PMA is associated with a rapid loss of proliferative capacity.

(d) Eosinophils: Fischkoff *et al* . (1984) first noted that the proportion of cells exhibiting eosinophilic characteristics in the HL60 line could be increased markedly by culturing the cells in slightly alkaline media (pH 7.6-7.8). These cells demonstrate multiple eosinophilic granule proteins, including the major basic protein (MBP). Parental HL60 cells also exhibit somewhat lower levels of eosinophil MBP, but the amount diminishes even further when they are treated with granulocytic inducers such as DMSO (Fischkoff *et al.*, 1984). Some of the characteristics exhibited by HL60-derived 'eosinophils' are aberrant compared to normal eosinophils. The 'eosinophilic granules' of the HL60-derived cells, for instance, are chloroacetate esterase and PAS positive, whereas normal eosinophilic granules are negative for both (Fischkoff *et al.*, 1984). HL60 eosinophils express significantly less arylsulphatase protein than do normal eosinophils (Fischkoff *et al.*, 1986). Such abnormalities, which are observed also in cells from the bone marrow of patients with acute myelomonocytic leukaemia, are associated with the chromosome 16 abnormalities which have also been noted in HL60 cells (Fischkoff *et al.*, 1986).

1.9.2 Aims of the current study :

Significant clinical advances have been made towards the cure of neoplasia with existing chemotherapeutic agents, but the mechanism of action of these drugs is not selective and causes the cytodestruction of both normal and neoplastic cells. This suggests that other approaches to cancer therapy should be sought that do not involve killing cells. One such approach would be the conversion of malignant cells, through induced differentiation, to benign forms with no proliferative potential. The possible use of differentiation as a therapeutic approach is predicated on the basis that neoplasia is a disease of altered maturation. Such a phenomenon can be visualized in leukaemia where cells arrested at different maturational levels are regenerated continually by an active stem cell compartment. The carcinogen, which might be a proto-oncogene, may be visualized as interfering with the normal progression of a developing cell to its fully-differentiated end

stage with retention of its infinite proliferative capability. The therapeutic objective in this situation would be to 'push' cells beyond the block in maturation so that they progress to a more mature cellular stage that lacks proliferative capability. This could be achieved, in principle, by the use of differentiating agents in chemotherapy and, hence, in the treatment and cure of leukaemia.

The aim of this project is to measure the major metabolic changes which occur during the chemically-induced differentiation of leukaemic HL60 cells to non-tumourous granulocytes, monocytes and macrophages. Its purpose is to determine whether the inducing agents used (DMSO, butyrate and PMA) have any clinical potential to regulate the growth and proliferation of these neoplastic cells. Even though the use of DMSO and PMA as differentiating agents has long been known, their potential in chemotherapy has not been explored because of their cytotoxicity at the concentrations at which they induce differentiation. However, using these agents *in vitro* can help to unravel the complexities associated with proliferation/differentiation. Butyrate-induced partial remission of acute myeloid leukaemia in a child has been reported (Novogrodsky *et al.*, 1983), but the use of butyrate as a therapeutic drug is not well established, possibly due to the complex and poorly understood nature of its mechanism of action (Smith, 1986). The present investigation was carried out with a view to addressing the following questions.

(1) Are DMSO, butyrate and PMA capable of inducing HL60 cells to differentiated granulocytes, monocytes and macrophages ?

(2) To what extent do these differentiating agents convert neoplastic HL60 cells to cells that are phenotypically comparable to authentic control cells separated from human peripheral blood?

(3) Are the differentiated cells as capable as their parent cells of developing tumours in nude mice ? If they are, are the cells in tumours similar to HL60 cells ? This test is also a

measure of the extent to which differentiation has been brought about by DMSO, butyrate and PMA.

(4) Do the differentiated cells (in a study focussed on DMSO-differentiated cells only) have the same glycolytic and glutaminolytic pattern of metabolic activity as their parent cells ?

(5) Is the inhibition of proliferation in differentiated cells (DMSO-differentiated cells only) associated with the inhibition of purine and pyrimidine synthesis and, if so, is the purine biosynthetic pathway similar to that of appropriate control cells separated from peripheral blood ?

(6) Are the anti-metabolites which inhibit key enzymes of the purine and pyrimidine biosynthetic pathways also capable of inducing differentiation of HL60 cells ? These anti-metabolites (e.g. acivicin, alanosine, etc) are presently being used clinically as chemotherapeutic agents. A detailed knowledge of the biochemical mechanisms of these anti-metabolites is essential for the development of rational and effective chemotherapy. With this objective, experiments have been designed to provide insights into the mechanism by which some of these anti-metabolites induce differentiation in HL60 cells, and thus stop proliferation. These procedures would enable us to identify the sites of inhibition in nucleotide metabolism and, thus, to determine the 'metabolic cross-over points' where intermediates prior to and after the drug-induced blockade accumulate and become depleted, respectively, indicative of decreased flux through the reaction.

Chapter 2: Degranulation and production of reactive oxygen intermediates in untreated and dimethylsulphoxide, phorbolester and butyrate-treated HL60 cells

2 Introduction

The human promyelocytic leukaemic cell line designated HL60 has been widely studied as a model of myeloid differentiation (Newburger *et al.*, 1979). This cell line, established by Collins *et al.* (1977), is especially intriguing because the majority of the cells which proliferate as promyelocytes retain a latent commitment to myeloid maturation beyond the promyelocyte stage that can be evoked by external chemical agents. For example, when exposed to dimethylsulphoxide (DMSO) (Collin *et al.*, 1978), HL60 cells lose their proliferative potential and show sharply decreased expression of oncogene products (*c-myc* and *c-myb*) (Birnie, 1988). After 5-7 days in DMSO, the HL60 cells develop characteristic neutrophilic morphology. In contrast, exposure of HL60 cells to PMA (Rovera *et al.*, 1979) or sodium butyrate (Boyd and Metcalf, 1984) induces differentiation to a stage indistinguishable from cells of the monocytic lineage. They express monocytic cell surface antigens and a level of lysosomal enzyme activity consistent with their state of differentiation. Both the monocytoid and neutrophilic end products of HL60 cell differentiation acquire the ability to form reactive oxygen intermediates (ROI) as determined by the superoxide dismutase-inhibitable reduction of cytochrome C or the reduction of nitroblue tetrazolium dye (NBT) (Harris and Ralph, 1985).

In this study we have used DMSO and sodium butyrate to differentiate and induce HL60 cells to granulocytic/monocytic pathway, and PMA to differentiate them to macrophage-like cells. The molecular mechanism by which cellular differentiation is controlled to these pathways by the above compounds is still not clear. However, it is thought that one family of the molecules may control the events of differentiation and proliferation by acting on cellular genes and signal transduction molecules like protein kinases (e.g. DMSO and PMA) (Collins, 1987) while the other group initiates differentiation/proliferation by acting directly on DNA (e.g. DMSO and sodium butyrate) (Terada *et al.*, 1978 and Kruh, 1982).

Aim of the study

In the present study HL60 cells have been used as a model of selected haemic cell differentiation. Much is known about their phenotypic characteristics and their cell biology in response to DMSO, butyrate and PMA, but a detailed study of the time course of their differentiation has not been reported. Since these cells undergo differentiation to phagocyte-like cells (e.g. granulocytes, monocytes and macrophages) which play an important role in host defense mechanism involving phagocytosis-associated oxidative metabolism that leads to the production of ROI (Allen *et al.*, 1972) and, also involves non-oxidative processes like the secretion of lysosomal enzymes (Johnston *et al.*, 1976, Bretz and Baggiolini, 1974 and Steigbigel *et al.*, 1974), it was important to investigate their biochemical parameters. Hence, a detailed time course of differentiation was measured using morphological changes, the formation of ROI and the secretion of lysosomal enzymes as a convenient marker. ROI production (in the presence and absence of differentiaing agents) was measured by luminol-dependent chemiluminescence (CL), cytochrome C reduction and nitrobluetetrazolium dye reduction. The changes in the non-oxidative phagocytic properties of both the undifferentiated and differentiated cells were evaluated by measuring the activities of the lysosomal enzymes lysozyme, acid phosphatase, myeloperoxidase, specific esterase and non-specific esterase. Authentic polymorphonuclear leukocytes (PMNs) and mononuclear cells (MNs) were separated from human blood to serve as controls. All these experiments have been described in Sections 2.1.3 to 2.1.13. Since terminal-differentiation is involved with the cessation of proliferation this study also describes the extent of proliferation of HL60 cells in the presence and absence of differentiating agents. The proliferative capacity of the cells was evaluated by measuring the synthesis of DNA by the incorporation of [^{3}H]-thymidine (Section 2.2.14). Because of the asynchronous nature of HL60 differentiation, and due to the presence of differentiation-resistant population in the cell system, experiments were designed using a discontinuos Percoll gradient to quantify and separate the fully differentiated cells from the less differentiating cell population (Section 2.1.15). This

method would supposedly separate the denser differentiated cells from the less denser undifferentiated ones.

The above experiments were designed to assess the extent of differentiation of HL60 cells with each of the differentiating agent used. The results would provide interesting and useful information about the phenotypic and biochemical changes associated with differentiation to non-proliferative granulocytes, monocytes and macrophages.

2.1 Materials and Methods

2.1.1 Chemicals

PBS was obtained from Flow Laboratories (Victoria, Australia). Dimethylsulphoxide was obtained from Fluka AG (Germany). Sodium butyrate, paranitrophenol, osmic acid, sodium cacodylate and 2-(4'-tert-butylphenyl)-5-(4''-biphenylyl)-1,3,4,oxadiazole (butyl-PBD) were from BDH Chemicals (U.K). Phorbol 12-myristate 13-acetate, superoxide dismutase (SOD), catalase, horseradish peroxidase (HRP), lysozyme, *Micrococcus lysodicticus*, α−naphthyl acetate, α−naphthyl butyrate and monochlorodimedon were obtained from Sigma Chemical Company (U.S.A.). Cytochrome C from horse heart, luminol and p-nitrophenyl phosphate were purchased from Boehringer Mannheim (Germany). Uranyl acetate was obtained from Ajax Chemicals, Sydney, Australia, and Spurr's low viscosity resin kit was bought from Bio-Rad Company (U.S.A). Organic solvents and inorganic chemicals, all of analytical grade, were obtained from Univar Chemicals, Sydney, Australia; BDH Chemicals, UK; and May and Baker, Australia Pty Ltd. Distilled water was used to prepare all buffers and solutions.

2.1.2 Cells

Human promyelocytic HL60 leukaemic cells were obtained from Dr J.Hamilton, Royal Melbourne Hospital, Victoria, Australia. The cells were grown in suspension culture and passaged twice weekly in 75 cm^2 tissue culture flasks in RPMI-1640 medium (Commonwealth Serum Laboratories, Victoria, Australia) supplemented with 20% foetal

calf serum (Commonwealth Serum Laboratories, Victoria, Australia), penicillin (60μg/ml), streptomycin (100 μg/ml), glutamine (2mM) and sodium pyruvate (1mM). The cells were grown at 37^0C in a humidified atmosphere of 5% CO_2 and 95% air. After 6-10 passages, aliquots of cells (2 x 10^6/ml) were frozen in liquid nitrogen in a mixture containing glycerol (10%), foetal calf serum (30%) and RPMI-1640 medium (60%). Experiments were conducted on cells which had been maintained in culture for a period of only six months. After each six month period a new batch of frozen cells was put in culture and experiments were continued. The number of cells was determined using a haemocytometer, and cell viability was estimated using trypan blue dye exclusion. The authenticity of the cells was verified by their morphology and behavioural pattern as described in the literature (Collins *et al.*, 1978; Newburger *et al.*, 1979).

2.1.3 Differentiation of cells

For the induction of differentiation, HL60 cells were seeded at a density of $2x10^5$ - $3x10^5$ cells/ml and grown in the presence of either DMSO (1%), sodium butyrate (0.6mM) or PMA (100nM). For the morphological assessment of the cells, Cytospin slide preparations of 0.2ml aliquots of cell suspensions were made using a Shandon Southern Cytospin. The cells were stained with Diff-Quik [Lab Aids, New South Wales (NSW), Australia] which stains cells in a manner similar to the Wright-Giemsa stain. Morphological observations of the slides were made by light microscopy using oil at x 1000 magnification.

2.1.4 Electron microscopy

Morphological differences between the undifferentiated and differentiated cells were also assessed by electron microscopy. For this purpose the cells were collected into a pellet ($1x10^6$cells) by centrifuging at 1500 x g for 10 mins and subjected to primary fixation with glutaraldehyde (2%) in cacodylate buffer (0.1M, pH 7.4) for 2 hours. The samples were washed in cacodylate buffer (0.1M, pH 7.4) for one hour and post-fixed in osmium tetraoxide solution (1%) for 90 mins. The post-fixed samples were washed twice for 5 mins in cacodylate buffer, rinsed in distilled water, stained with uranyl acetate (1%) for

90 mins, washed again in distilled water (twice for 5 mins each) and dehydrated by exposure to successively higher concentrations of ethanol (30%, 50%, 70%, 90% and 95%) for 5-10 mins. Dehydrated samples were agitated twice (for 90 mins each time), first in a mixture of Spurr's resin/ absolute alcohol (1:1) and then in absolute Spurr's resin only. After agitation the samples were embedded and polymerized for 8 hours at -80°C, cut in ultra thin sections (100nm) on an Reichert-Jung ultracut E ultramicrotone and stained again with uranyl acetate and Reynold's lead citrate. The thin samples were examined in a Philips 301 transmission electron microscope and representative cells of each sample were photographed.

2.1.5 Isolation of polymorphonuclear leukocytes (PMNs) and mononuclear cells (MNs) from whole blood

Fresh venous blood (10-50 ml) was collected, in heparinized tubes, from healthy male donors (20-50 years of age) at the Red Cross Blood Bank, Woden Valley Hospital, Australian Capital Territory. Within one hour of blood collection, 5ml of blood was layered gently on top of 4ml of Mono-Poly Resolving Medium (Flow Laboratories, NSW, Australia) in a sterile 15ml centrifuge tube and centrifuged at 400 x g for 30 mins in a swinging-arm bench centrifuge at room temperature. The PMN and MN fractions were removed as soon as possible with a pasteur pipette and placed into sterile 50ml centrifuge tubes and washed three times in phosphate-buffered saline (PBS) (400 x g for 10 mins). If any red blood cell contamination remained in the pellets after the final wash, the pellets were resuspended in 25ml of sterile distilled water and the tubes were gently and repeatedly inverted for one minute. The hypotonic shock treatment was stopped by the addition of an equal volume of double-strength PBS and the normal washing procedure was resumed. After the final wash the cells were resuspended in 2-5ml of PBS, capped and stored on ice. The viability and concentration of the cells were determined using the trypan blue exclusion method. The purity of the cells was checked by centrifuging a 200µl sample ($5x10^5$ cells) onto a microscopic slide using a Cytospin and examining the smear microscopically after staining with Diff Quik .

2.1.6 Cytochrome C reduction

The rate of superoxide anion production was measured by discontinuous spectrophotometric assay of the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome C to the ferrous form as described by Markert *et al.* (1984). The superoxide anion production detected by this assay depends on its release into the extracellular medium where it reacts with ferricytochrome C.

$$Fe^{3+}\ Cyt\ C\ +\ O_2^{-\cdot} \rightarrow Fe^{2+}\ Cyt\ C\ +\ O_2$$

Cells (2×10^6) were incubated in 1ml of Hank's balanced salt solution (HBSS), pH 7.4, containing cytochrome C (120μM). The reaction was initiated by addition of PMA (1.5μg/ml) in the presence or absence of SOD (90U/ml). All incubations were performed at 37^0C for 10 mins. The reaction was stopped by placing the tubes on ice for 5 mins, after which the cells were removed by centrifuging at 12000 x g for 2 mins. The supernatant containing the reduced cytochrome C was measured at 550nm against a blank containing all reagents except the cells.

2.1.7 Luminol-dependent chemiluminescence (CL)

The generation of reactive oxygen intermediates, e.g., superoxide anions, hydrogen peroxide, singlet oxygen and hydroxyl radicals, as a result of respiratory burst activation in phagocytic cells is essential for luminescence (Section 1.8.1., Figs 1.10a, 1.10b and 1.10c). This phenomenon was first described by Allen *et al.*, (1972) and has since been a tool for the study, not only of the phagocytic function itself, but also for the complement system, as well as for the assay of substances likely to influence the activity of phagocytosis. Luminescence produced by the unstable reactive oxygen intermediates (e.g. $^1O_2$) as a result of its return to less energetic triplet state ($^3O_2$) is partially emitted as light in the red region of the spectrum. Most photomultiplier show poor sensitivity in this region of the spectrum and the detection of such luminescence requires sensitive luminometer. By the addition of high quantum yield organic substrates such as luminol (5-amino 2,3 dihydro 1,4 phthalazinedone) it has been possible to increase the sensitivity

of the method by several orders of magnitude (Fig 2.5) (Allen *et al.*, 1976 and Dyke *et al.*, 1977). As a result ~~of light emission~~ the mechanism of light emission of phagocytic cells due to respiratory burst activation changes from involving both O_2^- and myeloperoxidase (Rosen and Klebanoff, 1976) to being totally dependent on the myeloperoxidase-H_2O_2 system (Dahlgren and Stendahl, 1983 and DeChatelet *et al.*, 1982). Myeloperoxidase acts in a direct way by catalysing the production of HOCl (Fig 1.10c). In the presence of both H_2O_2 and HOCl, luminol will luminescence spontaneously. The chemiluminescence observed with luminol is depressed by the absence or inhibition of myeloperoxidase and also by the action of catalase which breaks down H_2O_2.

CL was measured using a 6 channel Hamilton Lumicon luminometer. Luminescence of the cells was estimated with luminol (220μM) in a total volume of 1ml of cell suspension ($1x10^6$ cells/ml) in the presence or absence of horseradish peroxidase (HRP, 9U/ml). HRP was added to compensate for the possible absence of myeloperoxidase in the cells. PMA (1μM) or heat-killed opsonized *Staphylococcus aureus* (0.2mg/ml) were used as stimulants and the luminescence was recorded until the maximum rate was achieved.

2.1.8 Preparation of opsonized heat-killed *Staphylococcus aureus*

S. aureus was seeded into 200ml of Luria broth and allowed to grow for 18 hours at 37°C. The bacteria were then pelleted and resuspended in 20ml of HBSS. Human serum (20ml) was added to the bacteria, which were placed in an oscillating water bath (100 oscillations/min) at 37°C for 40 mins. The bacteria were washed twice in HBSS, resuspended again in 20ml of HBSS and incubated in a water bath at 60°C for 1 hour. Bacteria were collected, resuspended in 20ml of HBSS, divided into 1ml samples and stored at -20°C. The protein concentration of the suspension was adjusted to 2mg/ml.

2.1.9 Nitroblue tetrazolium dye reduction

NBT reduction was measured as the formation of a black precipitate (formazan) in the cells formed by the reduction of NBT by superoxide anion:

$$NBT + O_2^{-\cdot} \rightarrow Formazan + O_2$$

NBT (100µl of 0.2%) was added to a suspension of 1 x 10^6 cells in a total volume of 0.5 ml, which were then incubated at 37^{0}C for 30 mins. Production of reactive oxygen intermediates was stimulated with PMA (1.5µg) and the final reaction volume was made to 1ml with HBSS. After 30 mins the reaction was stopped by placing the tubes on ice for 5 mins. The number of cells forming the black formazan precipitate was counted using a haemocytometer and expressed as a percentage of the total cell number.

2.1.10 Acid Phosphatase (AP) activity

AP activity was determined on the basis of hydrolysis of p-nitrophenyl phosphate in an acetate buffer, pH 5.3, as described by Metcalf *et al* . (1986a). 100µl of cell lysate (1x 10^6 cells) was used for each assay. The cell lysate was prepared by sonicating the cells in PBS, on ice, at a setting of 7, using an MSE 100 watt ultrasonic disintegrator for a total of 90 secs in three 30 sec pulses with an interval of 30 secs between each pulse.

2.1.11 Specific and Non-Specific Esterase (NSE) activity

Naphthol AS-D chloroacetate and α–naphthyl acetate were used as substrates in these assays. In these assays AS-D Chloro naphthol and naphthol are liberated by specific esterase and NSE and is coupled to a diazonium salt producing an insoluble, brightly coloured azo dye that stains human granulocytes and monocytes to a characteristic blue and brick red colour (Metcalf *et al.*, 1986b). These stains enable accurate quantitation of granulocytes or granulocyte-derived cells and monocytes or monocyte-derived cells. Specific esterase and NSE activities were measured as described in the Sigma Diagnostics Kit (Procedure No 91, Revised December 1988).

2.1.12 Lysozyme activity

Both intra and extra-cellular lysozyme activities were measured using *Micrococcus lysodicticus* as a substrate, by the method described by Metcalf *et al.* (1986c).

Extracellular lysozyme was measured following the concentration of the medium 20-30 fold by ultrafiltration using Amicon PM-10 filters.

2.1.13 Myeloperoxidase (MPO) activity

Myeloperoxidase activity was measured, using monochlorodimedon as a substrate, by the method described by Winterbourn *et al.* (1985). 50-200µl of cell lysate prepared as described in Section 2.1.10 was used for each assay.

All protein measurements were made by the method of Lowry *et al.* (1951).

2.1.14 [^{3}H]-Thymidine incorporation

Five different lots of samples, each containing 1 x 10^6 cells/ml, were set up in a total of 2 ml of RPMI 1640 (containing 20% FCS). On day 2, 4, 6 and 8 respectively 0.2 µCi of [^{3}H]-thymidine was added to each sample. The cells were incubated at 37^0C in a 5% CO_2 and 95% air mixture for 16 hours, washed twice with PBS and once with 10% tricholoacetic acid. The precipitated pellet was dissolved in 0.1N NaOH (100µl) and [^{3}H]-thymidine incorporated into the cellular pellet was counted by liquid scintillation counting [using a mixture of toluene: methoxyethanol (6:4) containing 6g of butyl-PBD] using a Beckman scintillation counter model LS 2800. All counting was corrected for quenching by the addition of a known volume of an external standard of [^{3}H]-thymidine to each individual sample. The interference produced by the samples as a result of quenching was evaluated by the known counts of the external standard itself and corrected for the final counts which was expressed as disintegration/min (dpm).

2.1.15 Separation of DMSO-treated HL60 cells on Percoll density gradient.

DMSO-differentiated HL60 cells were separated on a Percoll density gradient as described by Foo (1987). Discontinuous Percoll gradients with densities of 1.088, 1.079 and 1.077 g/ml were set up in glass centrifuge tubes. Different Percoll densities were calculated using the following formula:

$$V_0 = V\,[(\rho-0.1)\,(\rho_{10}-0.9)/\,(\rho_0-1)]$$

where,

V_0 = Volume of Percoll from stock (ml)

V = Volume of final working solution (ml)

ρ = Desired density of final solution (g/ml)

ρ_0 = Density of stock Percoll (g/ml)

ρ_{10} = Density of 1.5M NaCl (1.058g/ml)

Three solutions of varying density (1.088, 1.079, 1.077 g/ml) were prepared according to the formula and kept on ice. Using cooled 10ml glass centrifuge tubes, the Percoll solutions (4ml) were layered carefully over each other, starting with the most dense solution and progressing through the successively lighter bands (Fig 2). The cell preparation containing 25 x 10^6 cells was layered gently on the top of the gradient and centrifuged at 4^0C for 20 mins at 600g. The fractions of the cells obtained were washed twice with PBS containing 5mM glucose, and the number and viability of cells determined using the haemocytometer and trypan blue dye exclusion.

2.2 Results

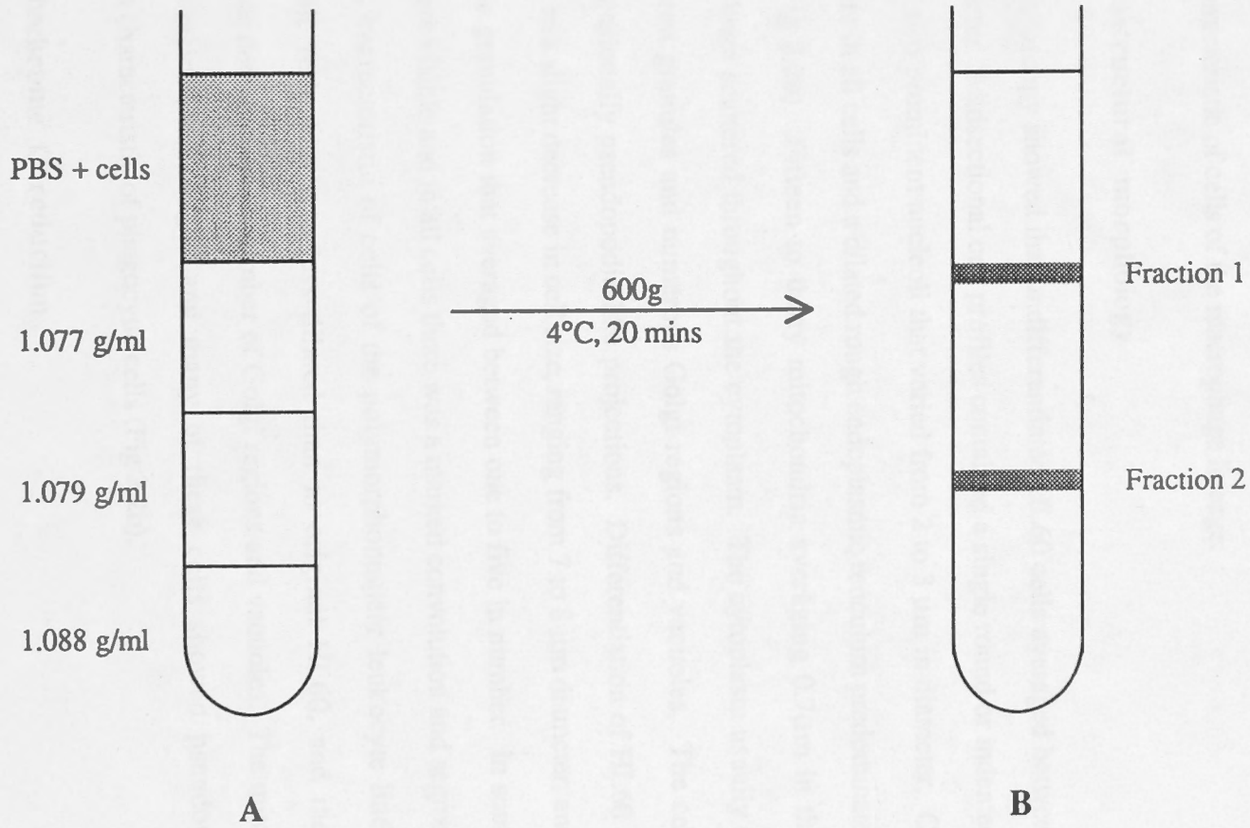
2.2.1 Light microscopy morphology

Undifferentiated HL60 cells are large, round cells with a high nuclear/cytoplasmic ratio, a blast-like nucleus and basophilic cytoplasm that contains numerous granules (Fig 2.1a). HL60 cells differentiated with DMSO (1%) decreased in size progressively over 5-7 days accompanied by a fall in the nuclear/cytoplasmic ratio, a trend towards less prominent cytoplasmic granulation and a marked reduction in or complete disappearance of nucleoli (Fig 2.1b). DMSO treatment also caused major changes to the gross morphology of the nucleus: within 5-7 days, 70% of the cells showed marked indentation, convolution and segmentation of the nuclei to an end-stage characteristic of polymorphonuclear leukocytes. HL60 cells treated with sodium butyrate (0.6mM) for 5-7 days became large, rounded cells with a small compact nucleus and a much less basophilic, but more

Fig 2:- Discontinuous Percoll gradient:

(A) Before and (B) After centrifugation.

Fig 2


PBS + cells
1.077 g/ml
1.079 g/ml
1.088 g/ml
600g
4°C, 20 mins
Fraction 1
Fraction 2
A
B

prominently vacuolated, cytoplasm than untreated cells. Binucleated and multinucleated giant cells were also observed occasionally. Within 5-7 days 50% of the cells had developed irregular or bean-shaped nuclei characteristic of cells of the monocyte/macrophage lineage (Fig 2.1c). Similarly, when HL60 cells were treated with 100nM PMA for 24 hours, they became large adherent cells with many cytoplasmic vacuoles characteristic of cells of the macrophage lineage.

2.2.2 Ultrastructural morphology

Electron microscopy showed that undifferentiated HL60 cells averaged between 9 to 11 μm in diameter. Midsectional cell profiles contained a single round or indented nucleus with one or two prominent nucleoli that varied from 2 to 3 μm in diameter. Cytoplasm was abundant in all cells and a dilated rough endoplasmic reticulum predominated in most of them (Fig 2.2a). Fifteen to thirty mitochondria averaging 0.7μm in the longest dimension were scattered throughout the cytoplasm. The cytoplasm usually contained five to fifteen granules and numerous Golgi regions and vacuoles. The cell surface showed occasionally pseudopodia-like projections. Differentiation of HL60 cells with DMSO led to a slight decrease in cell size, ranging from 7 to 8 μm diameter, and a loss of cytoplasmic granulation that averaged between one to five in number. In some cells no nucleoli were visible and in all cells there was a marked convolution and segmentation of the nuclei, characteristic of cells of the polymorphonuclear leukocyte lineage. The endoplasmic reticulum was less dilated than in original HL60, and there was a considerable decrease in the number of Golgi regions and vacuoles. The mitochondrial number remained unchanged and many of these cells showed pseudopodia-like projections characteristic of phagocytic cells (Fig 2.2b).

2.2.3 Cytochrome C reduction

Undifferentiated HL60 cells showed, on the basis of cytochrome C reduction, a very low rate of superoxide anion release, while HL60 cells differentiated for 9 days with DMSO (1%) released superoxide anion in response to PMA at an increased rate similar to that of peripheral blood neutrophils (Fig 2.4). The maximum rate of superoxide anion

Fig 2.1:- Light microscopy view of cells.

(**a**) HL60 and (**b**) 9 day DMSO-treated HL60 cells as seen by light microscopy (magnification x 1000).

Fig 2.1 (a)

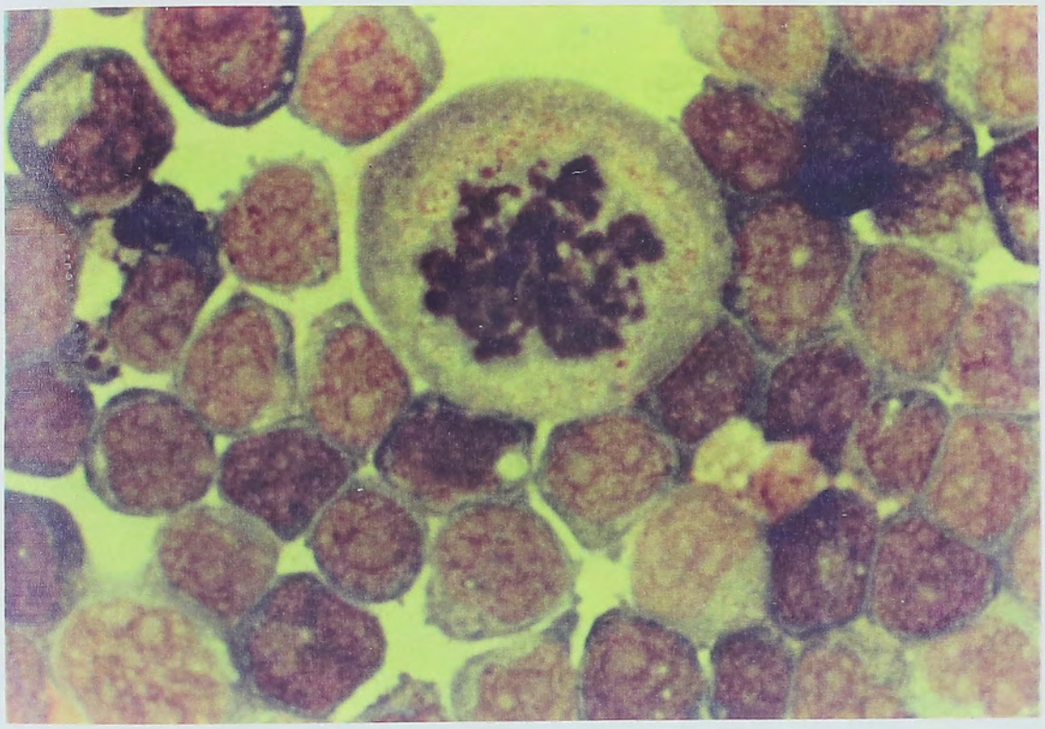


Fig 2.1 (b)

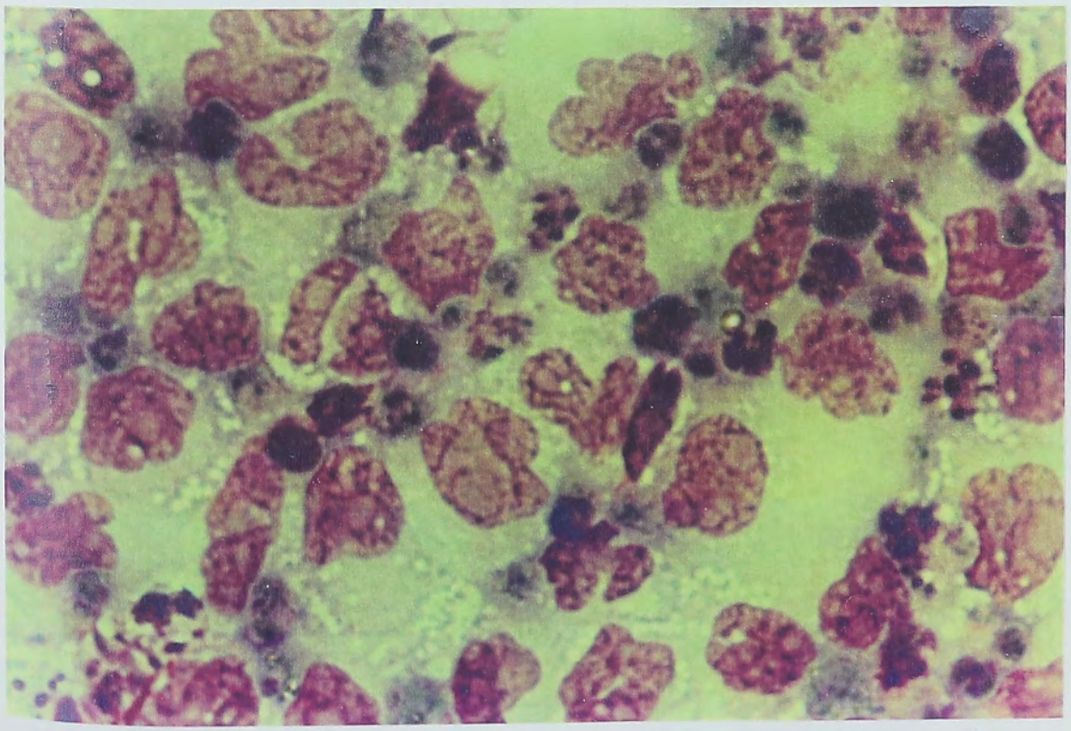


Fig 2.1:- Light microscopy view of cells.

(c) 9 day butyrate-treated HL60 cells as seen by light microscopy (magnification x 1000).

000).

Fig 2.1 (c)

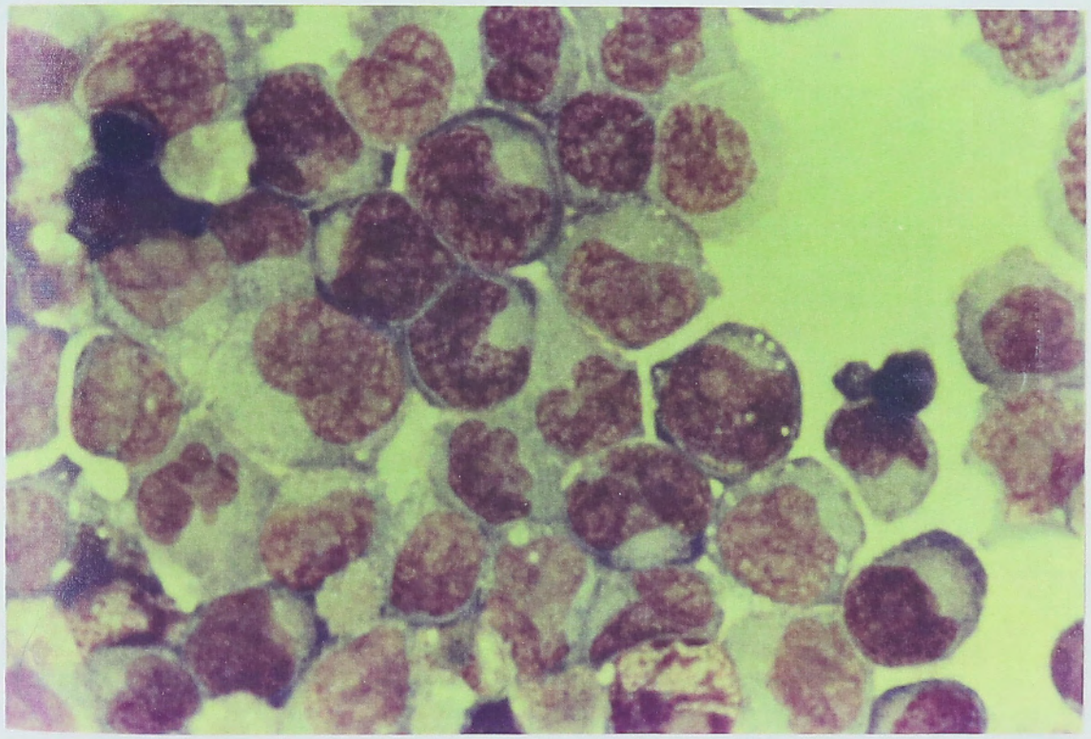


Fig 2.2:- Ultrastructural appearance of cells.

(a) HL60 (magnification x 10,000) and (b) 9 day DMSO-treated cells (magnification x 10,000).

Fig 2.2 (a)

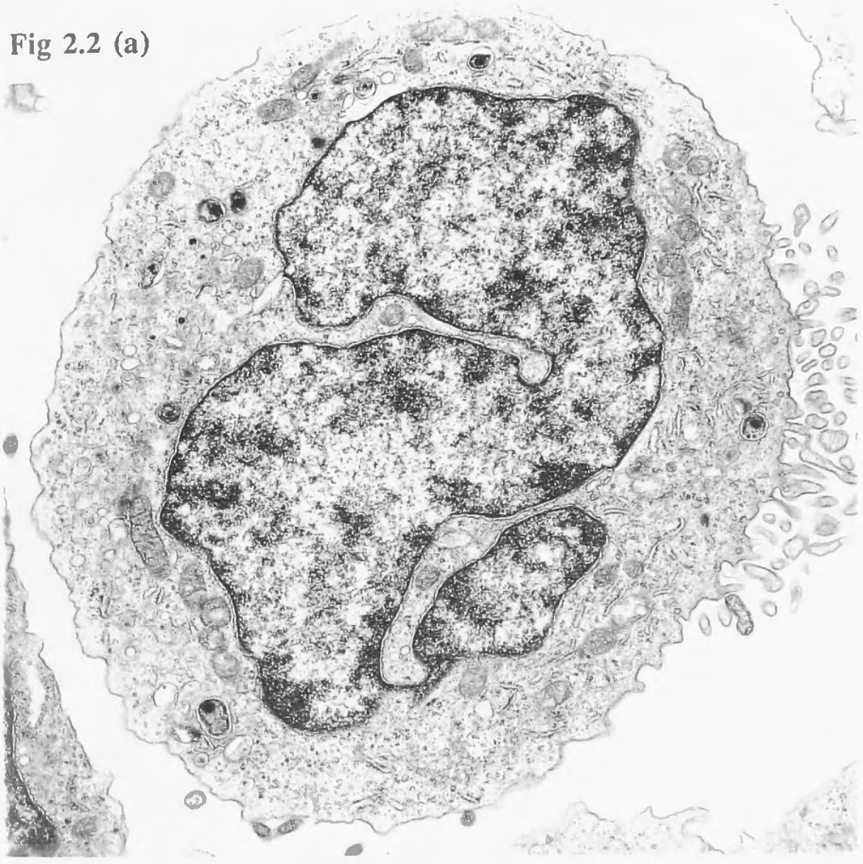


Fig 2.2 (b)

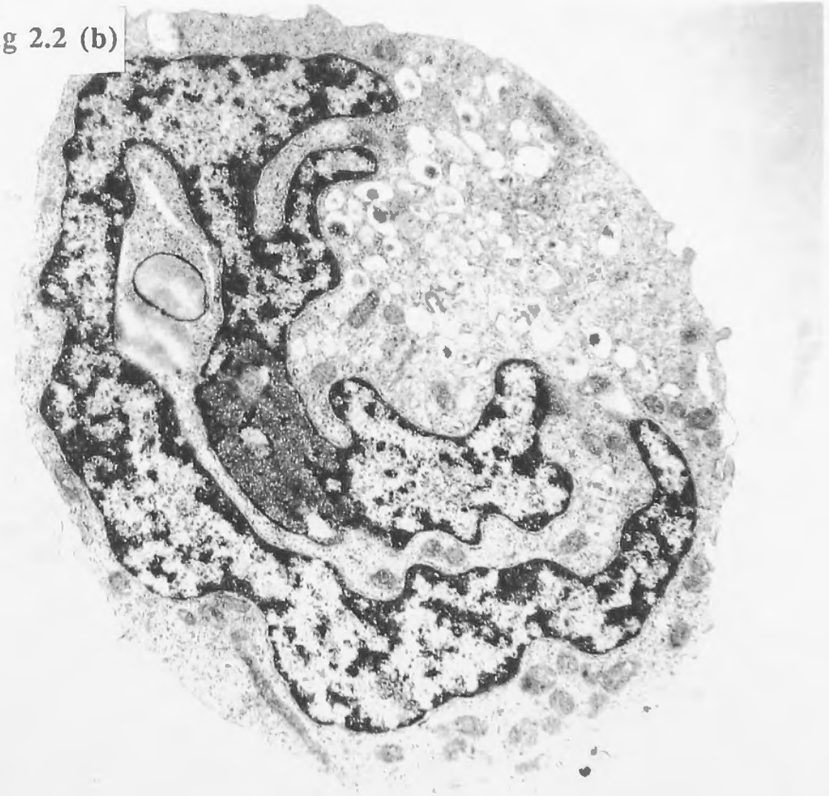


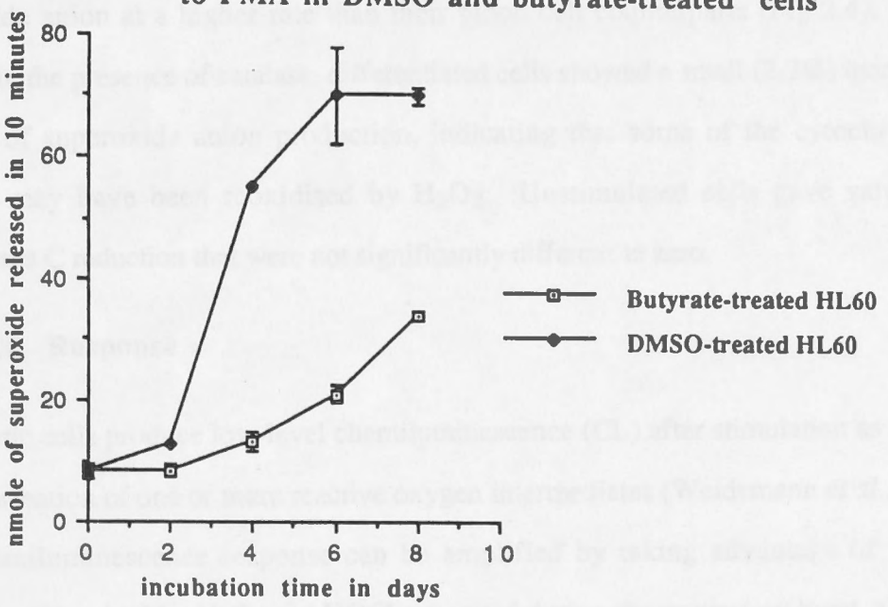
Fig 2.3 :- Time course of superoxide production in DMSO and butyrate-treated cells in response to PMA.

Data shown are the means and ± SD of three separate experiments, each performed in duplicate. Other details are given in the Materials and Methods section.

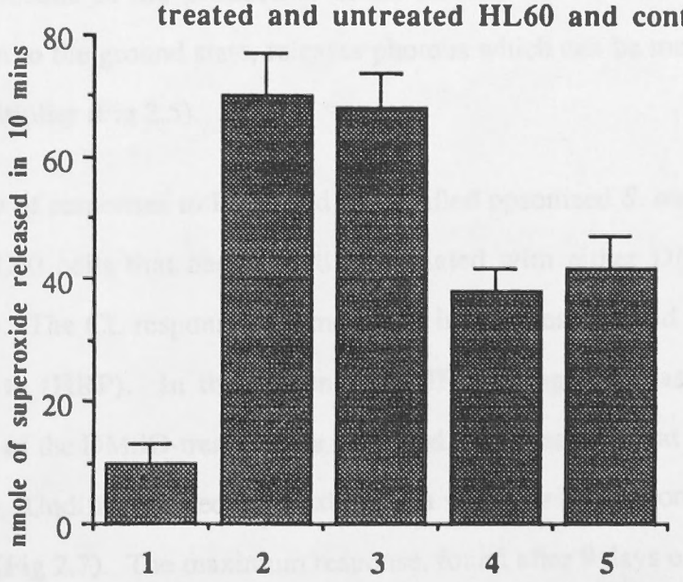
Fig 2.4 :- Superoxide production in response to PMA in treated and untreated HL60 and control cells.

1 (HL60), **2** (PMNs), **3** (9 day DMSO-treated cells), **4** (MNs) and **5** (9 day butyrate-treated cells). Superoxide production was determined by measuring the reduction of cytochrome C at 550nm. Superoxide production by unstimulated cells was not significantly different from 0. Data shown are the means and ± SD of three separate experiments, each performed in duplicate. Other details are given in the Materials and Methods section. **2**, **3**, **4** and **5** are significantly different from **1**, ($p < 0.01$).

(2.3) Time course of superoxide production in response to PMA in DMSO and butyrate-treated cells



(2.4) Superoxide production in response to PMA in treated and untreated HL60 and control cells



production occurred between 8-10 days exposure to the differentiating agents (Fig 2.3). Cells exposed to butyrate (0.6mM) released superoxide anion at a lower rate than the DMSO-differentiated cells. After 9 days, cells treated with butyrate (0.6mM) produced superoxide anion at a higher rate than their blood cell counterparts (Fig 2.4). When assayed in the presence of catalase, differentiated cells showed a small (2-3%) increase in the rate of superoxide anion production, indicating that some of the cytochrome C reduced may have been reoxidised by H_2O_2. Unstimulated cells gave values for cytochrome C reduction that were not significantly different to zero.

2.2.4 CL Response

Phagocytic cells produce low-level chemiluminescence (CL) after stimulation as a result of the formation of one or more reactive oxygen intermediates (Weidemann *et al.*, 1978). The chemiluminescence response can be amplified by taking advantage of the co-oxidation of luminol by H_2O_2 and HOCl generated during the respiratory burst, although the precise details of the chemical mechanism are not known. The co-oxidation of luminol results in the production of an excited aminophthalate anion which, upon relaxation to the ground state, releases photons which can be measured with a sensitive photomultiplier (Fig 2.5).

A variety of responses to PMA and heat- killed opsonized *S. aureus* CL was observed using HL60 cells that had been differentiated with either DMSO (1%) or butyrate (0.6mM). The CL response was measured in the presence and absence of horseradish peroxidase (HRP). In the presence of HRP, using PMA as the stimulus, the CL response of the DMSO-treated cells increased 6-7 times over that measured in its absence (Fig 2.6). Undifferentiated cells exhibited a very low CL response even in the presence of HRP (Fig 2.7). The maximum response, found after 9 days of DMSO treatment (Fig 2.8), was approximately the same as that exhibited by human neutrophils in the presence of HRP (Fig 2.7). In the absence of HRP the response of the treated cells was much lower than that found in control human neutrophils (Fig 2.6). Similarly for butyrate-treated cells, using PMA as a stimulus, the maximum response in the presence of HRP

Fig 2.5:- Schematic description of the proposed mechanism to describe luminol-mediated chemiluminescence.

Fig 2.5

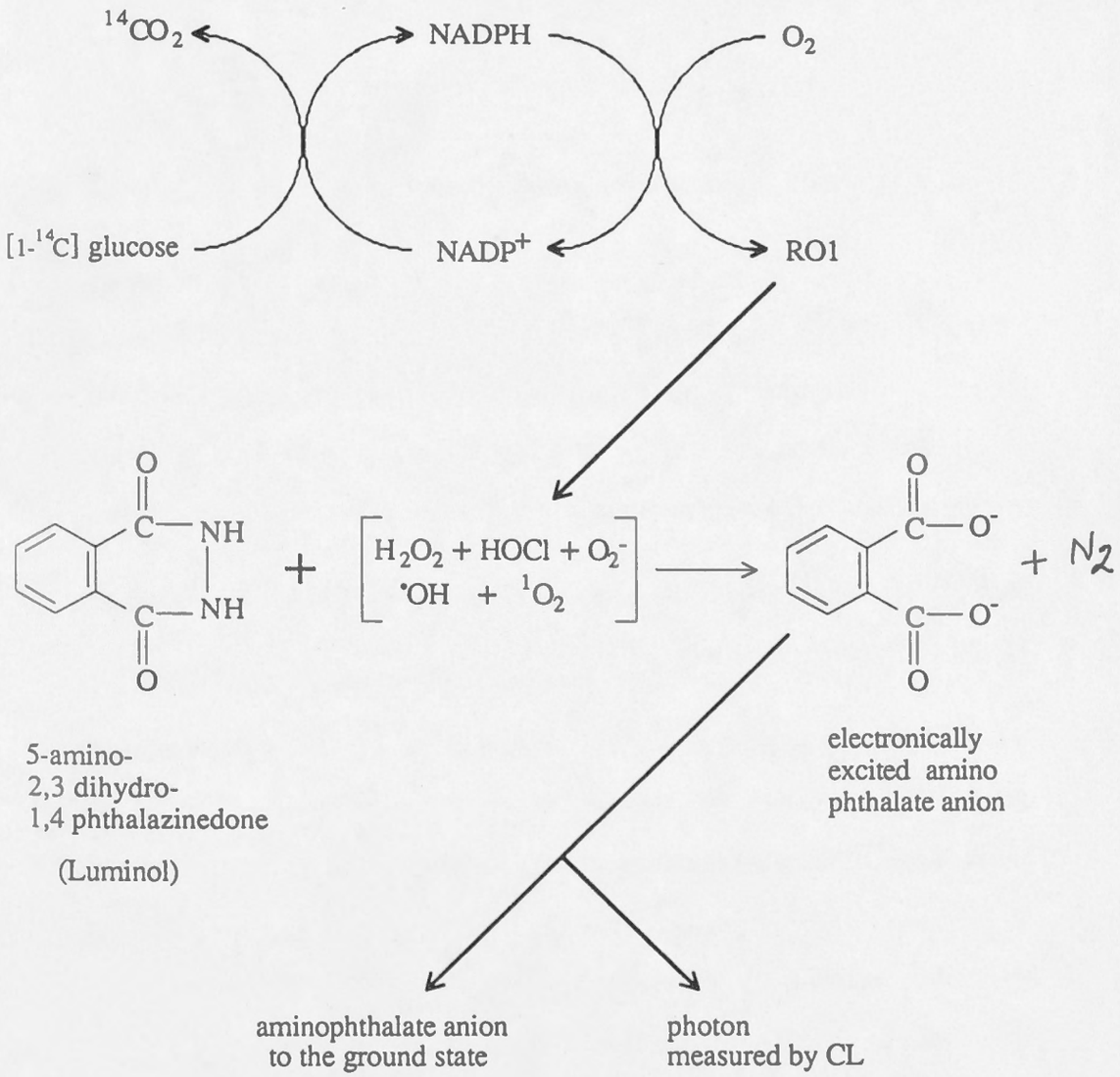

$^{14}CO_2$
NADPH
O_2
[1-^{14}C] glucose
$NADP^+$
RO1
$H_2O_2 + HOCl + O_2^-$
$^{\bullet}OH + {}^1O_2$
+ N_2
5-amino-
2,3 dihydro-
1,4 phthalazinedone
(Luminol)
electronically
excited amino
phthalate anion
aminophthalate anion
to the ground state
photon
measured by CL

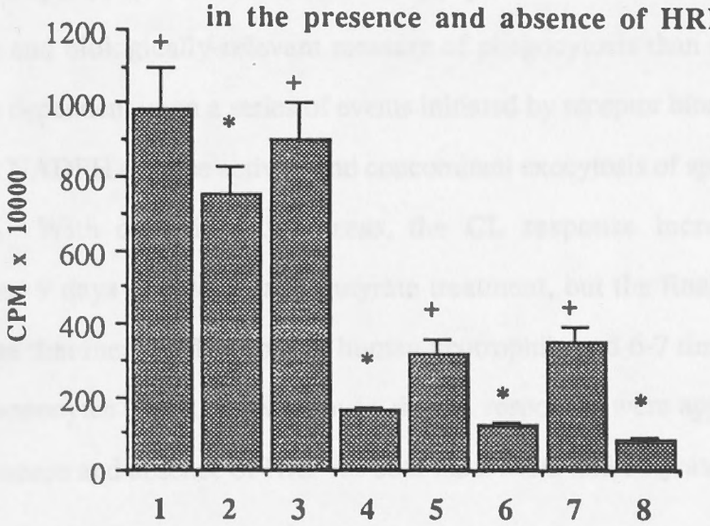
Fig 2.6 :- Peak chemiluminescence reponse to PMA in the presence and absence of HRP

(**1,2**) PMNs, (**3,4**) 9 day DMSO-treated cells, (**5,6**) MNs and (**7,8**) 9 day butyrate-treated cells. The experimental conditions are described in the Materials and Methods section. Data shown are the means and ± SD of three separate experiments, each performed in duplicate. (+) Presence of horseradish peroxidase, (*) Absence of horseradish peroxidase.

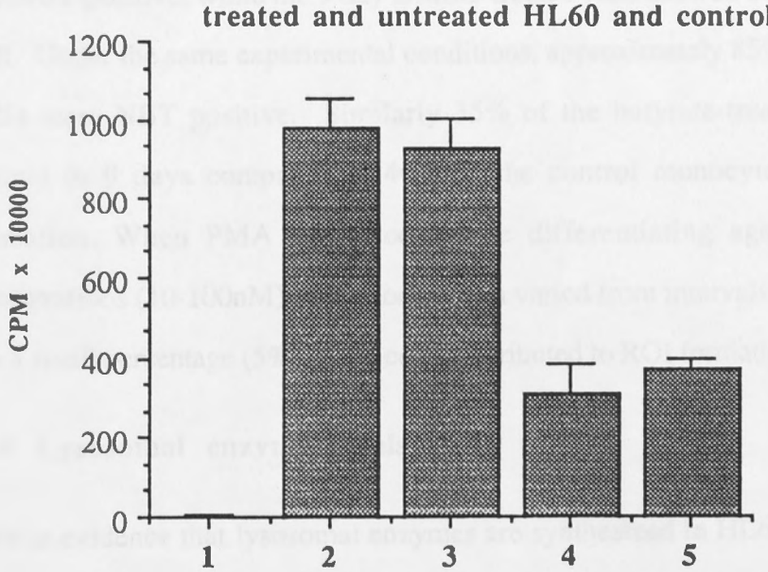
Fig 2.7 :- Peak chemiluminescence response to PMA of treated and untreated HL60 and control cells

1 (HL60), **2** (PMNs), **3** (9 day DMSO-treated cells), **4** (MNs) and **5** (9 day butyrate-treated cells). The CL response was measured in the presence of luminol (220μM) and HRP(9U/ml) using PMA (1μM) as the initiating stimulus. Light emitted by the unstimulated cells was not significantly different from that of the incubation medium in the absence of cells. Data shown are the means and ± SD of three separate experiments, each performed in duplicate. **2**, **3**, **4** and **5** are significantly different from **1**, ($p < 0.01$).

(2.6) Peak chemiluminescence response to PMA in the presence and absence of HRP



(2.7) Peak chemiluminescence response to PMA of treated and untreated HL60 and control cells



was observed by 9 days (Fig 2.8). The CL response was much greater in DMSO-treated cells than in butyrate-treated cells (Figs 2.7 and 2.8).

The CL response of cells stimulated with opsonized *S. aureus* represents a more complete and biologically-relevant measure of phagocytosis than that evoked by PMA since it is dependent upon a series of events initiated by receptor binding that culminate in increased NADPH oxidase activity and concomitant exocytosis of specific and azurophilic granules. With opsonized *S. aureus,* the CL response increased progressively throughout 9 days of DMSO and butyrate treatment, but the final value was 14 times lower than that measured in control human neutrophils and 6-7 times lower than that of human monocytes (Fig 2.9). .Moreover, the CL responses were approximately the same in the presence and absence of HRP for both the DMSO and butyrate-treated cells.

2.2.5 Nitroblue tetrazolium dye reduction

When NBT reduction was used to identify the particular cells capable of producing reactive oxygen species, it was found that only 5% of the undifferentiated HL60 cells were NBT positive, while the 9 day DMSO-treated cells showed 55% positive cells (Fig 2.10). Under the same experimental conditions, approximately 85% of authentic human PMNs were NBT positive. Similarly 35% of the butyrate-treated cells were NBT positive in 9 days compared to 40% of the control monocytes isolated from the circulation. When PMA was used as the differentiating agent over a range of concentrations (10-100nM) for periods which varied from intervals of 20 mins to 4 days, only a small percentage (5%) of the cells contributed to ROI formation.

2.2.6 Lysosomal enzyme levels

There is evidence that lysosomal enzymes are synthesized in HL60 cells, stored within granules as zymogens, and converted to active enzymes in response to the challenge of external stimulation (Yourno *et al.*, 1983)). Apart from acid phosphatase the levels of these enzymes in the differentiated HL60 cells were much lower than in their authentic blood cell controls (Table 2). The acid phosphatase activity reached a plateau between 8-

Fig 2.8 :- Time course of CL response in DMSO and butyrate-treated HL60 cells.

The CL response was measured in the presence of luminol (220μM) and ± HRP using PMA as the stimulating agent. Other details are given in the Materials and Methods section. Data given are the means ± SD of three separate experiments, each performed in duplicate.

(2.8) Time course of CL response to PMA in the presence and absence of HRP in DMSO and butyrate-treated cells

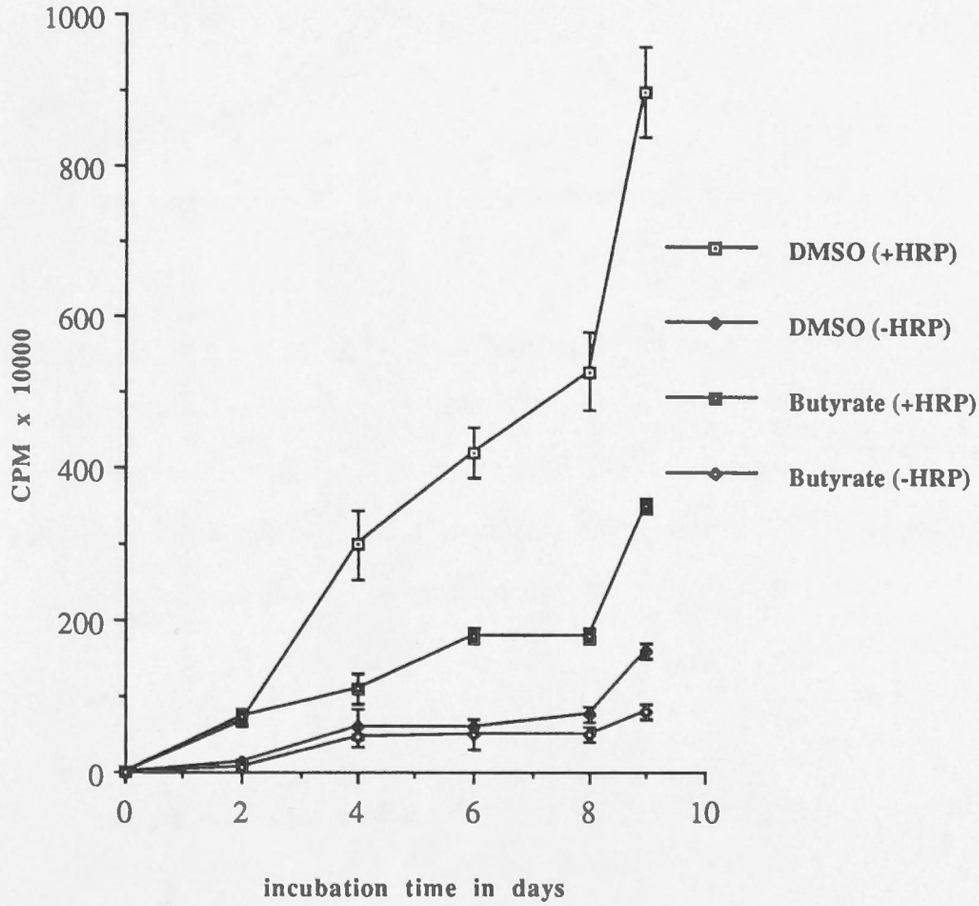


Fig 2.9 :- Peak chemiluminescence response to *S.aureus* of treated and untreated HL60 and control cells

1 (HL60), **2** (PMNs), **3** (9 day DMSO-treated cells), **4** (MNs) and **5** (9 day butyrate-treated cells). The CL response was measured in the presence of luminol (220μM) and HRP (9U/ml) using opsonized S. *aureus* (0.2mg/ml) as the stimulus. Light emitted by the unstimulated cells was not significantly different from that of the incubation medium in the absence of cells. Data shown are the means and ± SD of three separate experiments, each performed in duplicate.

(2.9) Peak chemiluminescence response to S. aureus of treated and untreated HL60 and control cells

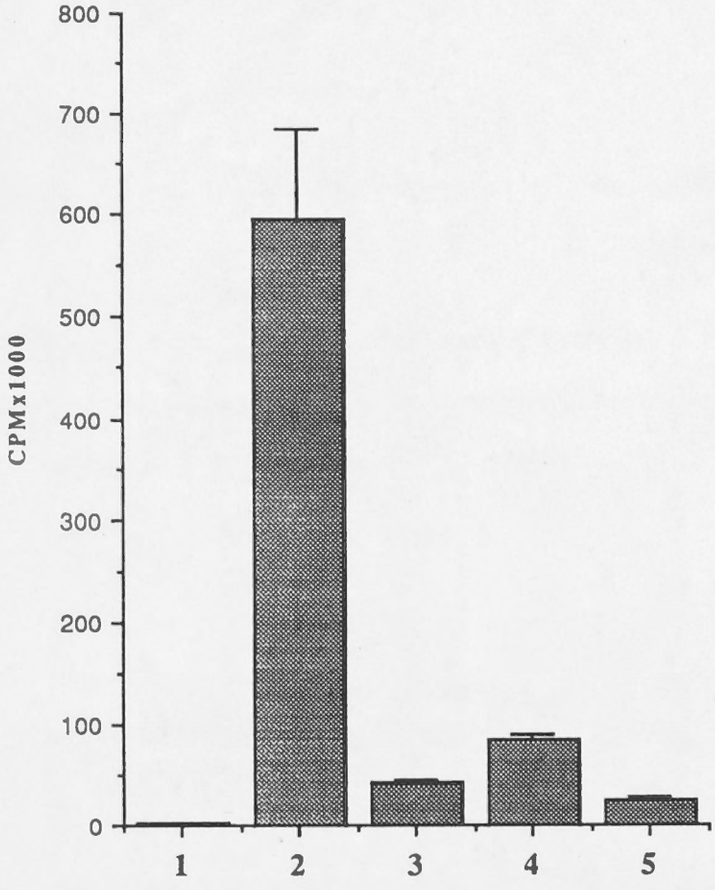
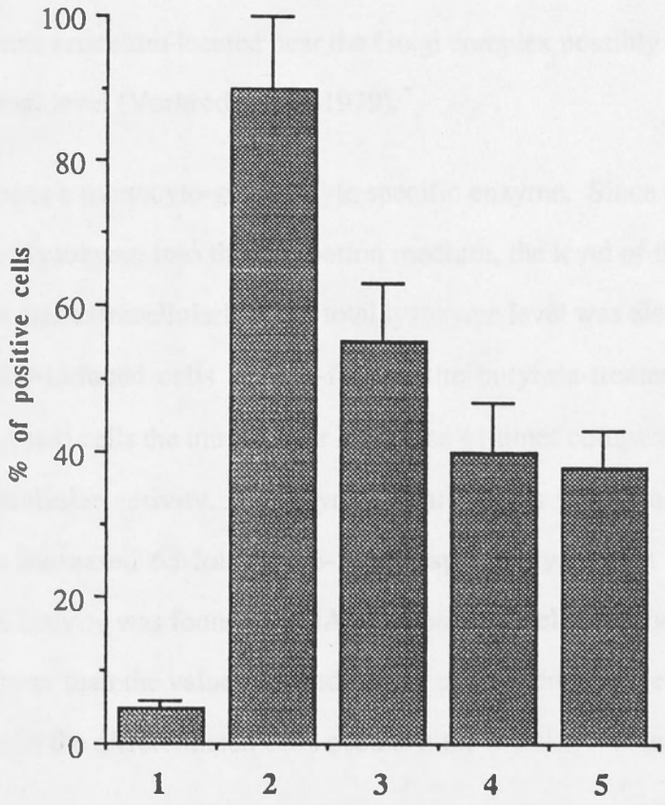


Fig 2.10 :- NBT reduction in response to PMA by treated and untreated HL60 and control cells

1 (HL60), **2** (PMNs), **3** (9 day DMSO-treated cells), **4** (MNs) and **5** (9 day butyrate-treated cells). NBT reduction was measured by counting the number of positive cells forming black formazan in response to PMA (1.5μg/ml). Other details are given in the Materials and Methods section. Data given are the means ± SD of three separate experiments, each performed in duplicate. **2, 3, 4** and **5** are significantly different from **1**, ($p < 0.01$).

(2.10) NBT reduction in response to PMA by treated and untreated HL60 and control cells



12 days in both DMSO and butyrate-differentiated cells (Fig 2.11). The levels achieved in these treated cells were approximately the same as those measured in the appropriate controls (Table 2). In PMA-differentiated cells the level of acid phosphatase showed a tendency to rise as early as 24 hours, and reached a maximum in 4 days (Table 2). The induction of acid phosphatase in differentiated HL60 cells is not precisely known but ultrastructural cytochemistry of DMSO and PMA-differentiated cells have shown this enzyme to be localized in the rough endoplasmic reticulum and in areas of the smooth endoplasmic reticulum located near the Golgi complex, possibly indicating induction at the translational level (Vorbrodt *et al.*, 1979).

Lysozyme is a monocyte-granulocyte specific enzyme. Since monocytes secrete a large amount of lysozyme into the incubation medium, the level of this enzyme was measured both intra and extracellularly. The total lysozyme level was elevated 48-fold by 6 days in the DMSO-induced cells and 72-fold in the butyrate-treated cells (Fig 2.12). With DMSO-treated cells the intracellular level rose 41 times compared with a 8-fold increase in the extracellular activity. In butyrate-treated cells the intracellular and extracellular activities increased 65-fold and 6-fold, respectively, within 6 days. No intracellular lysozyme activity was found in PMA-differentiated cells and the extracellular activity was always lower than the value reported for the undifferentiated cells (Table 2). Induction of lysozyme in the differentiated cells occurs at the translational level (Ralph *et sl.*, 1976).

Myeloperoxidase makes an important contribution to O_2-dependent microbicidal processes in neutrophils and is present at very high activity. Myeloperoxidase can also be demonstrated in monocytes. A low but finite activity of this enzyme was detected in HL60 cells but it was 4.5 times lower than that of authentic neutrophils. Myeloperoxidase was undetectable in DMSO and PMA-differentiated cells and was only present at negligible levels in butyrate-induced cells (Table 2).

Specific esterase (SE) is a characteristic marker enzyme for cells of the granulocytic lineage. Whilst more than 50% of the PMNs separated from human blood were SE positive, only 15% of the 6 day DMSO-treated cells stained for this activity. Although

Table 2: Lysosomal enzyme levels in treated and untreated HL60 and control cells

Cell Type	Specific-Esterase (% of positive cells)	Nonspecific-Esterase (% of positive cells)	Acid Phosphatase (μmole/10^6 cells)	Total Lysozyme (μg/10^6 cells)	Myeloperoxidase (μmole/10^6 cells)
HL60	5±0.5	5±0.5	0.04±0.00	0.01±0.00	0.04±0.00
PMNs	50±1.8	ND	0.14±0.02+	5.30±0.16+	0.18±0.00+
6 day DMSO-treated cells	15±0.9	9±0.5	0.12±0.00+	0.48±0.00+	0.0
MNs	ND	80±0.9	0.07±0.00+	2.15±0.05+	0.01±0.00
6 day butyrate-treated cells	5±0.5	48±1.8	0.08±0.00+	0.72±0.03+	0.03±0.00
1 day PMA-treated cells	16±1.9	35±2.0	0.06(*)	0.001(*)	0.0
4 day PMA-treated cells	ND	ND	0.12(*)	0.003(*)	0.0

All data are the means ± SD of at least three separate experiments, each performed in duplicate. (*) The values are the results of one representative experiment. ND means not determined. (+) Significantly different from HL60 cells, $p<0.01$.

Fig 2.11 :- The change in the acid phosphatase activity with time in DMSO and butyrate-treated HL60 cells.

The details of the experiment are given in the Materials and Methods section. Data given are the means $\pm$ SD of three separate experiments, each performed in duplicate.

(2.11) Time course of changes in acid phosphatase activity in DMSO and butyrate-treated cells

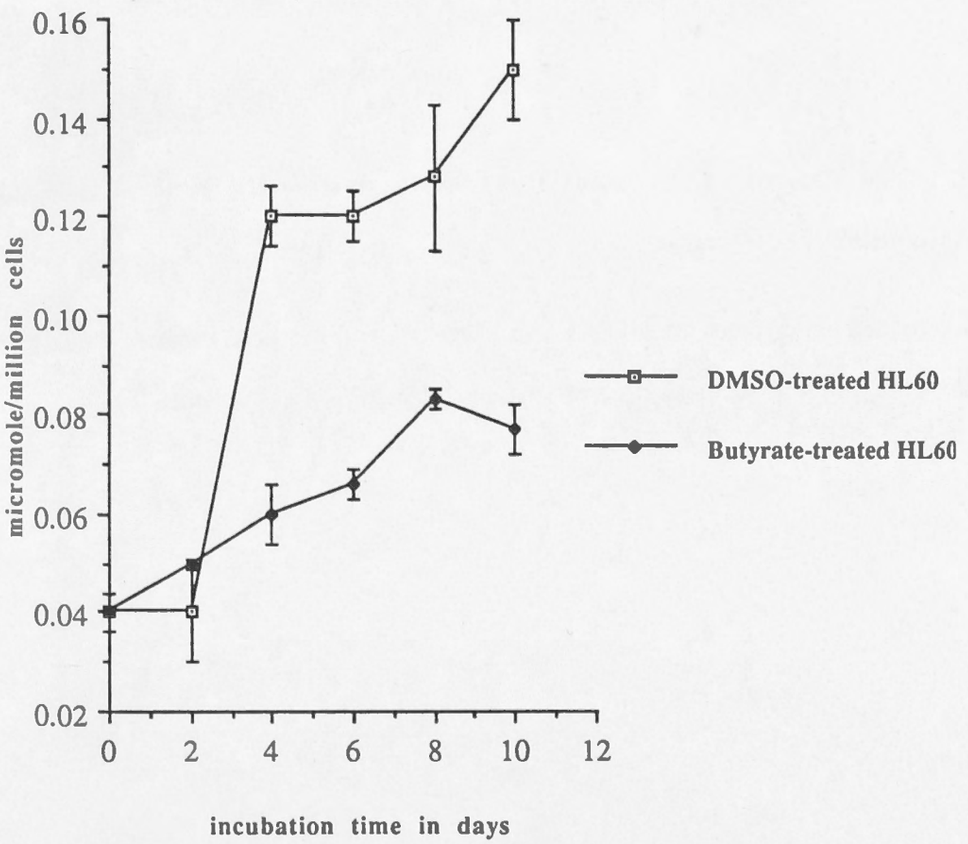
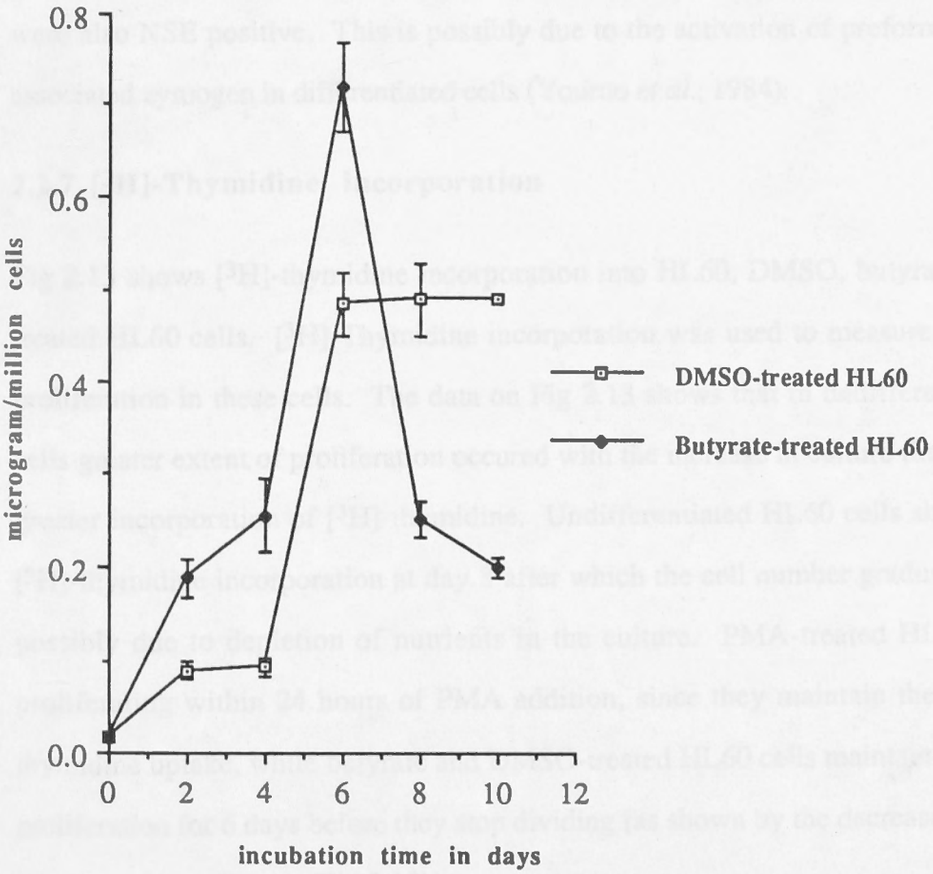


Fig 2.12: The change in the total lysozyme activity with time in DMSO and butyrate-treated HL60 cells.

The details of the experiment are given in the Materials and Methods section. Data given are the means ± SD of three separate experiments, each performed in duplicate.

(2.12) Time course of changes in lysozyme activity in DMSO and butyrate-treated cells



this enzyme should be absent from cells of the monocytic lineage, 5% of the 6 day butyrate-induced cells and 16% of the 1 day PMA-differentiated cells were SE positive. Conversely, non-specific esterase (NSE), which is detected primarily in monocytes, macrophages and histiocytes, is virtually absent from authentic granulocytes (Yamada and Kurahashi, 1984). About 80% of the MNs separated from blood were NSE-positive compared with 48% of the 6 day butyrate-treated cells and 35% of the 1 day PMA-induced cells. Unexpectedly, a significant proportion (9%) of the DMSO-treated cells were also NSE positive. This is possibly due to the activation of preformed, myeloid-associated zymogen in differentiated cells (Yourno *et al.*, 1984).

2.2.7 [^{3}H]-Thymidine incorporation

Fig 2.13 shows [^{3}H]-thymidine incorporation into HL60, DMSO, butyrate and PMA-treated HL60 cells. [^{3}H]-Thymidine incorporation was used to measure the extent of proliferation in these cells. The data on Fig 2.13 shows that in undifferentiated HL60 cells greater extent of proliferation occured with the increase in culture time resulting in greater incorporation of [^{3}H]-thymidine. Undifferentiated HL60 cells show a peak of [^{3}H]-thymidine incorporation at day 8 after which the cell number gradually decreases possibly due to depletion of nutrients in the culture. PMA-treated HL60 cells stop proliferating within 24 hours of PMA addition, since they maintain the same rate of thymidine uptake, while butyrate and DMSO-treated HL60 cells maintain a high rate of proliferation for 6 days before they stop dividing [as shown by the decrease in thymidine incorporation at 8 days (Fig 2.13)].

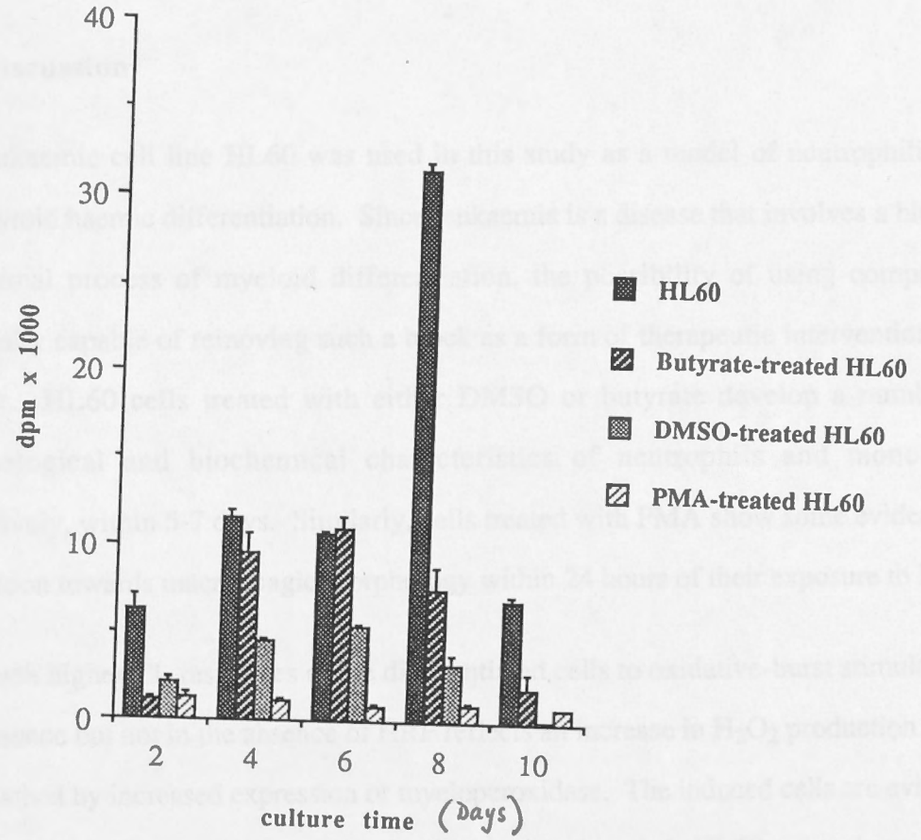
2.2.8 Separation of DMSO-treated HL60 cells on a density gradient

By means of density gradient centrifugation in Percoll, separations can be achieved of myelopoietic cells at various stages of differentiation (Olofsson *et al.*, 1980); the density of the cells increases with increasing maturation. HL60 cells induced to differentiate with DMSO for 6 days showed one major fraction, containing 50% of the cells loaded onto the gradient, while the rest of the cells – scattered throughout the gradient – were discarded. The CL response and superoxide production of the cells separated on the Percoll gradient

Fig 2.13 : [^{3}H]-Thymidine incorporation by untreated, DMSO, butyrate, and PMA-treated HL60 cells.

The details of the experiment are given in the Materials and Methods section. Data given are the means $\pm$ SD of three separate experiments, each performed in duplicate.

(2.13) Thymidine incorporation in untreated and DMSO, butyrate and PMA-treated HL60 cells



was significantly different from unseparated 6 day DMSO-treated HL60 cells ($p < 0.01$) (Table 2.1). HL60 cells treated for more than 6 days with DMSO gave two distinct fractions on Percoll. The low density fraction (Fraction 1) contained 80% of the cells loaded onto the gradient, in contrast to 20% of the cells recovered in the high density fraction (Fraction 2). Cells from the high density fractions had the same CL response and produced superoxide anion at the same rate as the 9 day DMSO-treated HL60 cells (Table 2.1) ($p=1.00$). On the other hand, cells from the low density Percoll fraction had a lower rate of ROI production (1.5 fold) than the 9 day DMSO-treated cells ($p< 0.01$).

2.3 Discussion

The leukaemic cell line HL60 was used in this study as a model of neutrophilic and monocytoid haemic differentiation. Since leukaemia is a disease that involves a block in the normal process of myeloid differentiation, the possibility of using compounds potentially capable of removing such a block as a form of therapeutic intervention is of interest. HL60 cells treated with either DMSO or butyrate develop a number of morphological and biochemical characteristics of neutrophils and monocytes, respectively, within 5-7 days. Similarly, cells treated with PMA show some evidence of a transition towards macrophagic morphology within 24 hours of their exposure to PMA.

The much higher CL responses of the differentiated cells to oxidative-burst stimulants in the presence but not in the absence of HRP reflects an increase in H_2O_2 production that is not matched by increased expression of myeloperoxidase. The induced cells are evidently unable to synthesize myeloperoxidase at a higher rate than their HL60 progenitors, which is consistent with the observed absence of translatable mRNA for myeloperoxidase (Weil *et al.*, 1987). The higher response to stimulation in the presence of HRP compensates for the lack of myeloperoxidase activity by facilitating the direct catalysis of luminol oxidation by H_2O_2. By contrast, authentic neutrophils are rich in myeloperoxidase, which accounts for 3 to 5% of the dry weight of the cells (Klebanoff, 1980), and they show no significant increase in CL in the presence of HRP (Fig 2.6). In differentiated cells, the CL response when opsonized *S. aureus* was used as the stimulant was found to be the

Table 2.1: Characteristics of DMSO-treated cells separated on discontinuous Percoll gradient.

Cell Type	Superoxide production (nmole/2x10^6 cells)	CL response (+) HRP (CPM/10^6 cells)	CL response (-) HRP (CPM/10^6 cells)	% of cells having PMNs-like nucleus
6 day DMSO-treated cells	53.49±0.00	6.3±0.81	0.96±0.17	ND
6 day DMSO-treated cells separated on Percoll gradient	72.30±1.89	7.1±0.44	0.52±0.02	ND
9 day DMSO-treated HL60 cells	69.00±0.58	9.3±0.07	1.20±0.04	60-70
9 day DMSO-treated HL60 cells separated on Percoll gradient (Fraction 1)	48.20±1.20	7.0±0.98	1.10±0.56	50-60
9 day DMSO-treated HL60 cells separated on Percoll gradient (Fraction 2)	69.45±1.41	11.2±0.95	3.00±0.68	70-80

Cells were stimulated with PMA (1μM) and the CL response and superoxide production were measured as described in the Materials and Methods section. All data are the means ± SD of at least six determinations from two individual experiments.

same in the presence and absence of HRP and, overall, much less than in authentic neutrophils and monocytes, indicating that there may be a defect in the phagocytic process that is not evident when the CL signal is evoked by a phagocytosis-independent stimulus like PMA. The very low phagocytic activity may be due to a post-receptor defect in the receptor-mediated pathway, or because the receptors themselves are absent or defective. Undifferentiated HL60 cells are known to have very few F_c-IgG_1 and C_3b receptors (Harris and Ralph, 1985). Differentiation increases the number of these receptors (Mendelsohn *et al.*, 1980) but the increase may not reach a level where an adequate response in the receptor-mediated NADPH oxidase pathway can be evoked by stimuli that are opsonin-dependent.

The profile of lysosomal enzymes is a distinctive feature of fully-differentiated monocytes and granulocytes. This is illustrated by the results presented here, if it is assumed that DMSO-differentiated cells are neutrophil-like and butyrate-treated cells are monocyte-like in function. Apart from acid phosphatase, which is expressed moderately to strongly in both neutrophils and monocytes, the other two enzymes assayed (i.e. NSE and lysozyme) are present at higher activities in butyrate-treated than in DMSO-treated cells. The lower levels of the lysosomal enzymes in the differentiated cells compared with their control counterparts may be due either to the failure of the differentiating stimulus to increase the synthesis of specific granular constituents or to defective degranulation. The decrease observed in intracellular granule enzyme activity was corroborated by electron microscopy and may be explained by a 'dilution' of primary granules due to repeated cell division (Olsson and Olofsson, 1981). During maturation of promyelocytes to the myelocyte stage of differentiation, production of secondary granule lactoferrin is expected, especially as the differentiation of HL60 cells has been reported to be accompanied by the replacement of primary granules with smaller specific granules (Gallagher *et al.*, 1979). In fact, differentiated HL60 cells do not produce lactoferrin (Collins,1987) indicating that the production, assembly or composition of granules upon differentiation is abnormal. This is demonstrated by the absence of myeloperoxidase activity in the DMSO and PMA-differentiated cells and the presence of only minute levels

of the enzyme in butyrate-differentiated cells. The finding that the majority of the DMSO-differentiated cells do not shift to a higher cell density (indicative of fully-differentiated neutrophils) in Percoll indicates that the normal maturation process is not complete upon DMSO differentiation. The abnormalities in granule composition and cell density, which are probably related, may reflect peculiarities of the leukaemic origin of HL60 cells which cannot be reversed to a normal phenotype upon differentiation.

In this study the extent of proliferation was quantitated by measuring the incorporation of [^{3}H]-thymidine in the presence and absence of differentiating agents. The results of Fig 2.13 shows that PMA-differentiation is followed by abrupt cessation of DNA synthesis (measured by [^{3}H]-thymidine), while DMSO and butyrate-induced differentiation proceeds in the presence of DNA synthesis. HL60 cells exposed to butyrate express their differentiated phenotype by 6 days, indicating that this process does not lead to the inhibition of proliferation. This finding, which is consistent with the mode of monocytic differentiation induced by γ–interferon and retinoic acid, indicates that differentiation is not always accompanied, or followed, by inhibition of proliferative capacity (Ball *et al.*, 1984 and Hemmi and Breitman, 1987); indeed, in some cases, it might be followed by an enhancement of the proliferative capacity of the differentiated cells (Elias *et al.*, 1985) . Since DMSO-treated cells express their differentiated phenotype by 9 days, we can argue from the results presented in Fig 2.13 that granulocytic differentiation is followed by the inhibition of proliferation. The continuous proliferation of the HL60 cells in the presence of DMSO and butyrate is consistent with their long period of 'precommitment' before entering the committed phase (Yen, 1985). This might also explain the variation observed in the arrest of growth at different stages of the growth cycle when different differentiating agents are used. Since PMA initiates differentiation of HL60 cells by causing growth arrest directly during the S phase of the cell cycle, it is capable of inhibiting DNA synthesis and proliferation at time intervals as low as 20 mins after PMA addition (Collins, 1987). DMSO and butyrate-induced differentiation of HL60 cells involves inhibition of the G_1 phase of the growth cycle. As a result, cell cycle activity

and cell division continues during the early phase of differentiation before proliferation is stopped (Tarella, 1982; Boyd and Metcalf, 1984).

The present experiments included a study of PMA induction of HL60 cells in order to determine whether the differentiated phenotype, which is morphologically macrophage-like, conforms with the complete set of properties that are generally used to define a macrophage. Macrophages have such individual properties as: (1) a characteristic size (20-80 μm diameter); (2) low or absent proliferative capacity; (3) adherence to glass and certain plastic surfaces; (4) many lysosomal granules and high lysosomal enzyme activities, except for myeloperoxidase; and (5) synthesis of such biologically-active serum proteins as endogenous pyrogens (e.g. IL-1, tumour necrosis factor) and certain complement components. HL60 cells cultured in the presence of PMA met the first four of these criteria (Koeffler *et al.*, 1981 and Mendelsohn *et al.*, 1980). However, they showed no increase in respiratory burst activity (NBT reduction, superoxide anion production or increased CL response), suggesting that, although differentiation by PMA induces some macrophage-like properties, the cells failed to meet several of the additional criteria. Hence the system does not appear to be a suitable model for macrophage differentiation. PMA-induced cells should perhaps be termed 'macrophage-like' rather than macrophages for, although they share some phenotypic characteristics with macrophages, they lack some of the most important functions of mononuclear phagocytes (e.g. respiratory burst activity, C_2 or C_4 complement secretion, ingestion of complement-opsonized bacteria, etc).

Chapter 3: Differentiation of HL60 cells with dimethylsulphoxide; Production of reactive nitrogen intermediates

3 Introduction

Endothelium-derived relaxing factor (EDRF) is a labile, non-prostanoid humoral agent with a short half-life (3-50 secs) that is released from vascular endothelium in response to a variety of substances. It relaxes vascular smooth muscle, inhibits platelet aggregation, causes disaggregation of aggregated platelets and inhibits platelet adhesion. Its mode of action involves the activation of soluble guanylate cyclase and a resultant elevation of cGMP. It is inactivated by oxygen and superoxide anions and is bound with high affinity to haemoglobin. It is also inactivated by some redox compounds, possibly through the generation of O_2^- (Moncada *et al.*, 1988). Furchgott (1988) and Ignarro (1988) were the first to suggest that EDRF might be nitric oxide. Moncada *et al.* (1988) studied the comparative pharmacology of EDRF on vascular strips and platelets and demonstrated that nitric oxide is released from endothelial cells and vascular tissues in amounts sufficient to account for the biological activity observed. These and other studies showed that EDRF and nitric oxide were indistiguishable in terms of their chemical and pharmacological properties (Moncada *et al.*, 1988). It was shown later, by mass spectrophotometry, that endothelium-derived nitric oxide is synthesized enzymically from the amino-acid L-arginine (Palmer *et al.*, 1988). D-arginine could not substitute as a precursor when added along with L-arginine, each at 2mM concentration (Iyengar *et al.*, 1987). The following compounds were also found to be precursors [when compared with L-arginine, expressed as 100%] : L-homoarginine (80%), L-arginine methyl ester (82%), L-arginamide (72%) and the peptide L-arginyl-L-aspartate (84%) (Iyengar *et al.*, 1987). The synthesis of nitric oxide from L-arginine occurs through the action of an NADPH-specific enzyme (nitric oxide synthase) that requires a divalent cation, forms L-citrulline as a co-product and is inhibited by L-N^G-monomethyl arginine (L-NMMA) which is an enantiomeric-specific, competitive inhibitor of the enzyme (Palmer *et al.*, 1988; Palmer and Moncada 1989). Nitric oxide is very unstable under physiological conditions and is converted rapidly to nitrate and nitrite, which are stable intermediates. The nitrite formed is not derived from nitrate (Stuehr and Marletta, 1987); experiments

with [^{15}N]-arginine have shown that the source of nitrate and nitrite is one or both of the guanidino nitrogens of L-arginine (Iyengar *et al.*, 1987).

Nitric oxide is synthesized by many different cell types (e.g. endothelial cells, phagocytic cells like macrophages and neutrophils, brain cells, etc), and, although its overall pathway of formation in the different cell lines seems to be identical, there are marked differences in the particular enzymes involved. The major differences between the cell types seem to involve the *regulation* of the biosynthetic pathway, which may be related to the ultimate physiological function of the product. In phagocytic cells (macrophages and neutrophils), for example, no enzymatic activity is observed in freshly-isolated cells unless they have been activated by an authentic stimulus (e.g. PMA, LPS, IFN-γ, chemotactic peptide, etc). A lag phase, which is dependent on the concentration and nature of the activating agent, is observed, during which time protein synthesis required for the expression of the pathway enzymes takes place. The synthesis of nitric oxide occurs after the lag phase and continues linearly for some time, until cell death leads to its cessation (Stuehr and Marletta, 1987). The situation in endothelial cells is very different. Protein synthesis is not required. Stimulation by bradykinin, for example, leads to an immediate burst in nitric oxide synthesis which then decays rapidly (Palmer *et al.*, 1987). The rapid decay is due to reaction with O_2, leading to the production of nitrate and nitrite, typically in amounts that are below the limit of detection (Marletta, 1989). Experiments in the rat cerebellum have shown that the burst of nitric oxide formation that occurs after stimulation is similar to that observed in endothelial cells (Garthwaite *et al.*, 1988). The difference in the regulation of nitric oxide synthesis is due to the presence of two isoenzymic forms of nitric oxide synthase in the different cell types. The constitutive isoenzyme is Ca^{2+}, calmodulin and NADPH-dependent, and is present in endothelial and brain cells, while the inducible form, present in phagocytic cells, is Ca^{2+}-independent and requires tetrahydrobiopterin, flavin adenine dinucleotide and reduced glutathione, besides NADPH, as cofactors (Moncada and Higgs, 1991). L-NMMA inhibits both the constitutive and inducible enzymes. Nitric oxide is generated by the constitutive enzyme

for physiological purposes such as regulation of blood flow (e.g. in the endothelium and vascular tissue) and cell-to-cell communication (e.g. in the brain cells). The inducible enzyme generates nitric oxide for cytostatic/cytotoxic action directed against invading microorganisms, tumour cells, fungus, parasites, etc. The L-arginine:nitric oxide pathway is shown in Fig 3.

In Chapter 2 the formation of ROI have been described using luminol-dependent CL, cytochrome C reduction and NBT reduction. In this chapter the formation of RNI by nitric oxide synthase, acting in concert with ROI released by NADPH oxidase, is dealt with, using luminol-dependent CL, cytochrome C reduction and nitrate/nitrite measurements. Since RNI have been suggested to play a central role in macrophage-induced cytotoxicity (Albina *et al.*, 1989), the effects of intracellular signal molecules (e.g. PMA) which stimulate NADPH oxidase-catalyzed O_2^- formation in a plasma membrane receptor independent manner have been investigated on the formation and release of nitric oxide. The production and release of nitric oxide from human neutrophils, HL60 and DMSO-treated HL60 cells is reported in this Chapter. Due to limitations of time, only nitric oxide production by untreated and DMSO-differentiated HL60 cells has been investigated.

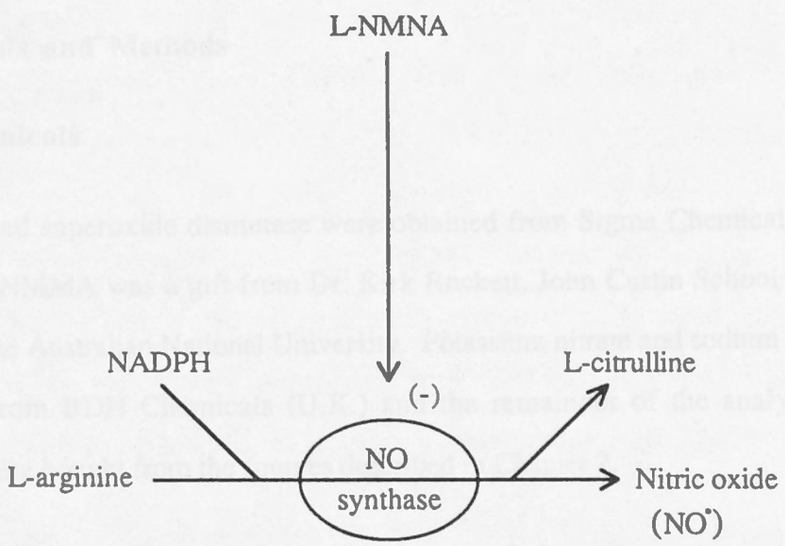
Aim of the study

HL60 cells undergo differentiation to phagocytic cells like neutrophils in the presence of DMSO. Since other phagocytic cells like macrophages are known to regulate the differentiation of haemopoietic cells in the bone marrow by secreting a variety of cytokines (Greenberger, 1991) it was important to evaluate the capacity of DMSO-differentiated HL60 cells for nitric oxide production in order to determine its correlation with cellular differentiation. Nitric oxide production by undifferentiated and DMSO-differentiated HL60 cells was detected using luminol-dependent CL, cytochrome C reduction and nitrate/nitrite measurements (Sections 3.13-3.1.5). Luminol-dependent CL and cytochrome C reduction methods measures the production of ROI. To assess the

Fig 3: Schematic diagram showing the synthesis of nitric oxide by the L-arginine-dependent pathway.

NO synthase can be either constitutive or inducible. L-NMMA can inhibit both the constitutive and inducible enzyme.

Fig 3


L-NMNA
NADPH
(-)
L-citrulline
NO
synthase
L-arginine
Nitric oxide
(NO•)

production and interaction of nitric oxide with ROI, L-arginine, a direct precursor of nitric oxide was used in these assays . Further assessment of nitric oxide production was made by using L-NMMA, an inhibitor of nitric oxide synthase, the enzyme responsible for nitric oxide production. Nitrate/nitrite were measured as the the end-product of nitric oxide excreted by the cells in the medium. Polymorphonuclear leukocytes separated from human blood were used as control in these experiments.

3.1 Materials and Methods

3.1.1 Chemicals

L-arginine and superoxide dismutase were obtained from Sigma Chemical Company, (U.S.A). L-NMMA was a gift from Dr. Kirk Rockett, John Curtin School of Medical Research, The Australian National University. Potassium nitrate and sodium nitrite were purchased from BDH Chemicals (U.K.) and the remainder of the analytical-grade chemicals were bought from the sources described in Chapter 2.

3.1.2 Cells

HL60 cell maintenance, and induction of differentiation by DMSO (1%) were performed as described in Chapter 2, Sections 2.1.2 and 2.1.3. Human PMNs isolated from blood obtained from a healthy adult female subject were separated on Mono-Poly Resolving Medium (described in Chapter 2, Section 2.1.5).

3.1.3 Effect of L-arginine and L-NMMA on luminol-dependent CL

Luminol-amplified chemiluminescence produced by HL60 cells, 9 day DMSO-treated cells and human PMNs was assayed in the presence and absence of L-arginine (0.1-20mM) and L-NMMA (0.1-10mM). The procedure described in Chapter 2 was followed except that no HRP was added. The reaction mixture contained luminol (220μM) in a total volume of 1ml of cell suspension (1×10^6 cells/ml in the case of HL60 and DMSO-treated cells; 5×10^5 cell/ml in the case of human PMNs). PMA (1μM) was used to

stimulate the cells and the luminescence was recorded until the maximum rate was achieved.

3.1.4 Effect of L-arginine and L-NMMA on cytochrome C reduction

The procedure for cytochrome C reduction for the measurement of O_2^- was the same as that described in Chapter 2, except that L-arginine (0.1mM-10mM) and, in some cases, L-NMMA (0.1mM-8mM) were added to the initial reaction mixtures. Cells (2×10^6 in the case of HL60 and DMSO-treated cells; 5×10^5 for human PMNs) were incubated in 1ml of HBSS, pH 7.4, containing cytochrome C (120μM). The reaction was initiated by addition of PMA (1.5μg/ml) in the presence or absence of SOD (90U/ml). All incubations were performed at 37°C for 10 mins. The reaction was stopped by placing the tubes on ice for 5 mins, after which the cells were removed by centrifuging at 12000 x g for 2 mins. The supernatant containing the reduced cytochrome C was measured at 550nm against a blank containing all reagents except the cells.

3.1.5 Estimation of Nitrate and Nitrite

For the estimation of nitrate and nitrite, cells were collected, washed twice with PBS and incubated (1×10^6/ml) at 37°C, in the presence of 5% CO_2 and 95% air, ±2mM arginine, ±3mM L-NMMA and ±120U of SOD for 30 mins. The reaction was initiated with the addition of PMA (1.5μg/ml). After 30 mins the reaction was stopped by transferring the tubes to ice for 10 mins. The supernates containing nitrate and nitrite were collected by centrifuging at 200 x g for 10 mins at 4°C. Nitrate was measured with a nitrate electrode, model 93-07 (Orion research incorporated, U.S.A.). A double-junction reference electrode, model 90-02, was used in conjunction with the main electrode. The sensitivity of the electrode was between 16μM to 2mM of nitrate concentration. The nitrate concentration in the samples was measured as millivolts by a millivolt meter (Specific ion, U.S.A.), and compared with a standard potassium nitrate curve constructed within the concentration range of 1-200mg/l. The desired concentrations of nitrate for the standard curve were obtained by diluting a stock potassium nitrate solution of concentration

5000mg/l. At this concentration nitrate was stable for several days. Nitrite in the samples was measured by the Griess reaction (Green *et al.*, 1982).

3.2 Results

3.2.1 Effect of L-arginine and L-NMMA on CL response

In undifferentiated HL60 cells , L-arginine concentrations up to 1mM had no effect on the luminol-dependent CL response to PMA (1μM). Concentrations of L-arginine above 1mM increased the peak CL response in a dose-dependent manner (Fig 3.1). The peak CL response in the presence of L-arginine was observed at 18-20 mins, while that for the generation of ROI in its absence occurred much earlier (at 3-5 mins). L-NMMA inhibited the L-arginine enhanced-response in a concentration-dependent manner. At 10mM L-arginine, 1mM L-NMMA showed no inhibition on the CL response ($p=1$), while 5 and 10mM L-NMMA, showed significant inhibition ($p<0.08$) (Fig 3.2). When the concentration of L-arginine was reduced to 2mM, 1mM and 0.5mM L-NMMA, this inhibited the peak responses by 40% in each case, while, in the case of 20mM arginine, the inhibition by 5 and 10mM L-NMMA was 40 and 80% respectively (results not shown in detail). L-NMMA added at different concentrations in the absence of any L-arginine had no effect on the luminol-dependent CL response ($p> 0.1$) (Fig 3.3). The slight inhibition observed at 5mM L-NMMA was not significant and is attributable to experimental error.

In 9 day DMSO-treated cells, L-arginine inhibited the luminol-dependent CL response to PMA in a dose-dependent manner, and the concentration required was approximately ten-fold lower than that used with HL60 cells (Fig 3.4). Furthermore, the peak CL response in the presence of L-arginine in 9 day DMSO-treated cells overlapped in time with that of the peak response observed with PMA alone (i.e. at 3-5 mins). L-NMMA enhanced the CL response in the presence of L-arginine by overcoming the arginine inhibition. When 1mM L-arginine was used, for example, L-NMMA at 0.1mM, 0.4mM, 0.8mM and 1.0mM concentrations significantly enhanced the peak CL responses ($p<0.02$) (Fig 3.5). Similarly, at 0.5mM L-arginine, L-NMMA at 0.1mM, 0.4mM and 0.5mM enhanced the

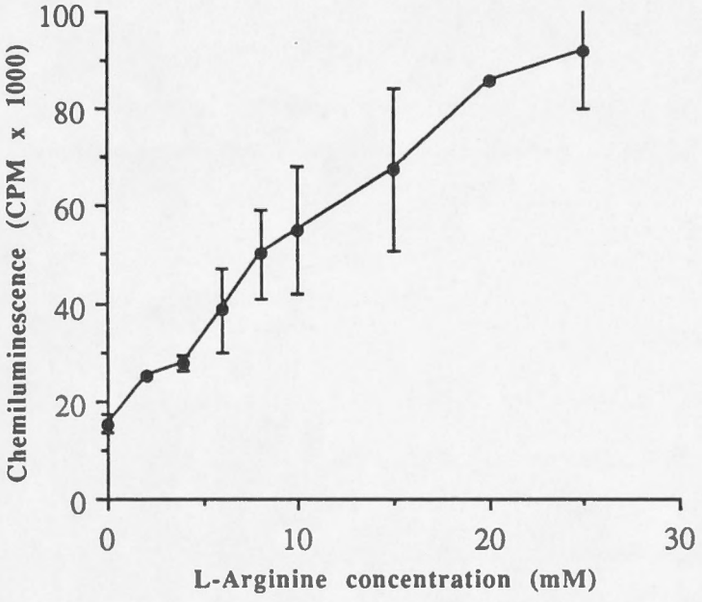
Fig 3.1: Peak CL response to PMA with increasing concentration of L-arginine in HL60 cells.

The conditions for the experiment are described in the Materials and Methods section. All data are the means ± S.E.M. of at least three separate experiments, each performed in duplicate.

Fig 3.2: Inhibition of peak luminol-dependent CL response to PMA in the presence of L-arginine (10mM) with increasing L-NMMA concentration in HL60 cells.

The conditions for the experiment are described in the Materials and Methods section. All data are the means ± S.E.M. of at least three separate experiments, each performed in duplicate. The CL response in the presence of 5 and 10mM L-NMMA is significantly smaller than that in the absence of L-NMMA, ($p < 0.08$).

(3.1) Peak CL response to PMA with increasing L-arginine concentration in HL60 cells



(3.2) Inhibition of the peak CL response to PMA in the presence of L-arginine (10mM) with increasing L-NMMA concentration in HL60 cells

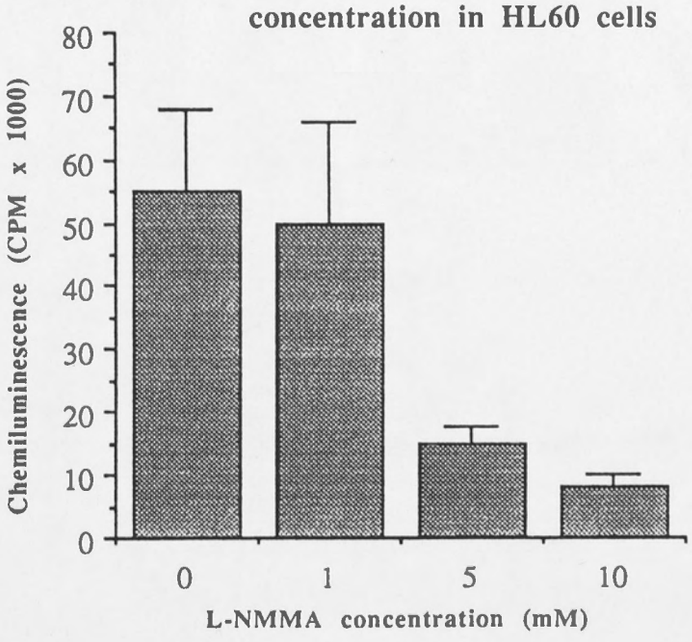


Fig 3.3: Effect of increasing the L-NMMA concentration on the peak CL responses to PMA in the absence of added L-arginine in HL60 cells.

The conditions for the experiment are described in the Materials and Methods section. All data are the means $\pm$ S.E.M. of at least three separate experiments, each performed in duplicate. The CL response in the presence of 1, 5 and 10mM L-NMMA is not significantly different than that in the absence of L-NMMA, ($p>0.1$).

(3.3) Effect of increasing L-NMMA concentration on the peak CL respones in the absence of added L-arginine in HL60 cells

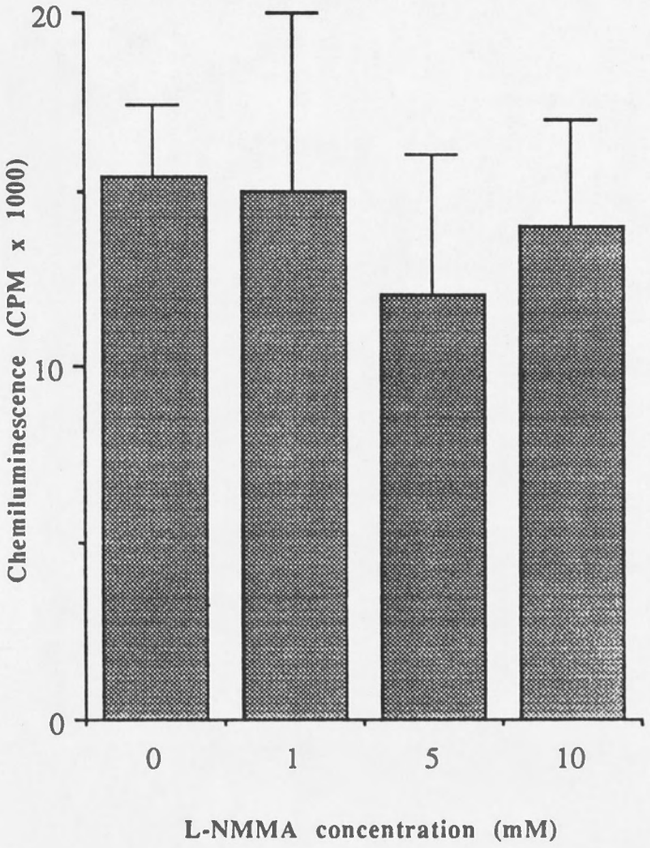


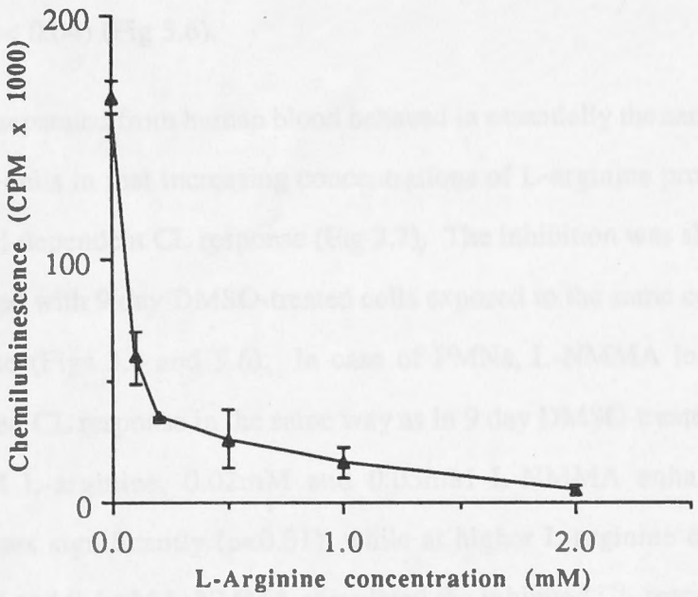
Fig 3.4: Peak CL response to PMA with increasing L-arginine concentration in 9 day DMSO-treated cells.

The conditions for the experiment are described in the Materials and Methods section. All data are the means ± S.E.M. of at least three separate experiments, each performed in duplicate.

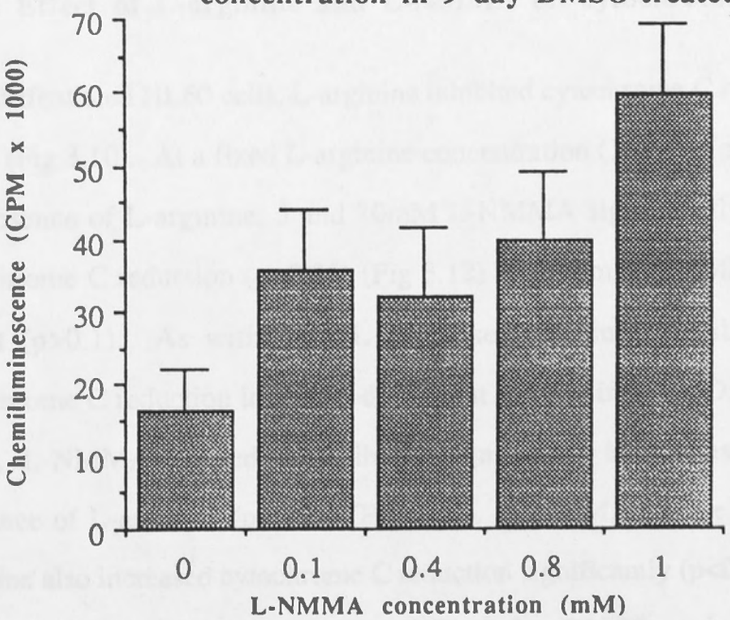
Fig 3.5: The enhancement of peak CL response to PMA in the presence of L-arginine (1mM) with increasing L-NMMA concentration in 9 day DMSO-treated cells.

The conditions for the experiment are described in the Materials and Methods section. All data are the means ± S.E.M. of at least three separate experiments, each performed in duplicate. The CL response in the presence of 0.1, 0.4, 0.8 and 1mM L-NMMA is significantly greater than that in the absence of L-NMMA, ($p<0.02$).

(3.4) Peak CL response to PMA with increasing L-arginine concentration in 9 day DMSO-treated cells



(3.5) Enhancement of peak CL response to PMA in the presence of L-arginine (1mM) with increasing L-NMMA concentration in 9 day DMSO-treated cells



response by 45% in each case (results not shown in detail). However, unlike HL60 cells L-NMMA alone, in the absence of L-arginine, inhibited significantly the luminol-dependent response in a concentration-dependent manner in the 9 day DMSO-treated cells ($p< 0.04$) (Fig 3.6).

PMNs separated from human blood behaved in essentially the same way as 9 day DMSO-treated cells in that increasing concentrations of L-arginine progressively inhibited the luminol-dependent CL response (Fig 3.7). The inhibition was slightly stronger than that observed with 9 day DMSO-treated cells exposed to the same concentration range of L-arginine (Figs 3.4 and 3.6). In case of PMNs, L-NMMA increased the L-arginine-inhibited CL response in the same way as in 9 day DMSO-treated cells (Fig 3.8); e.g., at 0.1mM L-arginine, 0.02mM and 0.05mM L-NMMA enhanced the inhibited CL responses significantly ($p<0.01$), while at higher L-arginine concentrations (0.5mM), 0.1mM and 0.4mM L-NMMA stimulated the inhibited CL responses by 92% and 100% (results not shown in detail). In the complete absence of added L-arginine, L-NMMA had no significant effect on luminol-dependent CL response in PMNs ($p>0.1$) (Fig 3.9).

3.2.2 Effect of L-arginine and L-NMMA on cytochrome C reduction

In undiffentiated HL60 cells, L-arginine inhibited cytochrome C reduction in response to PMA (Fig 3.10). At a fixed L-arginine concentration (10mM) ($p<0.02$)(Fig 3.11), or in the absence of L-arginine, 5 and 10mM L-NMMA significantly enhanced the rate of cytochrome C reduction ($p<0.05$) (Fig 3.12) while 1mM L-NMMA had no significant effect ($p>0.1$). As with the CL response, L-arginine inhibited PMA-stimulated cytochrome C reduction in a dose-dependent manner in 9 day DMSO-treated cells (Fig 3.13). L-NMMA relieved the inhibition significantly by increasing the response in the presence of L-arginine ($p<0.05$) (Fig 3.14). L-NMMA by itself in the absence of L-arginine also increased cytochrome C reduction significantly ($p<0.04$) (Fig 3.15). PMNs separated from human blood behaved like 9 day DMSO-treated cells with respect to cytochrome C reduction in the presence of L-arginine and L-NMMA (Fig 3.16).

Fig 3.6: Effect of increasing concentration of L-NMMA on the peak CL response to PMA in the absence of added L-arginine in 9 day DMSO-treated cells.

The conditions for the experiment are described in the Materials and Methods section. All data are the means ± S.E.M. of at least three separate experiments, each performed in duplicate. The CL response in the presence of 0.1, 0.4, 1.0 and 4mM L-NMMA is significantly smaller than that in the absence of L-NMMA, ($p<0.04$).

(3.6) Effect of increasing concentration of L-NMMA on the peak CL response to PMA in the absence of added L-arginine in 9 day DMSO-treated cells

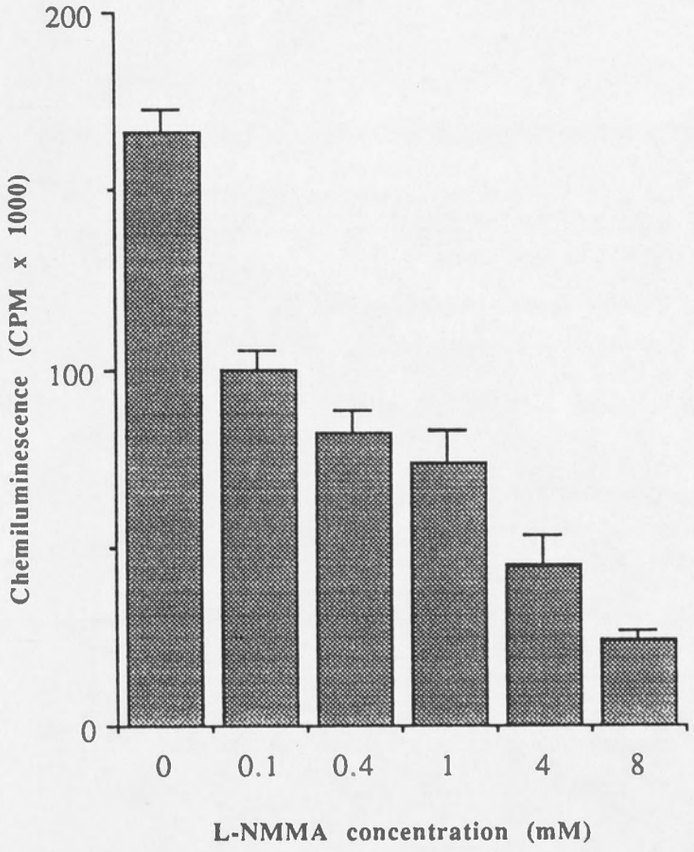


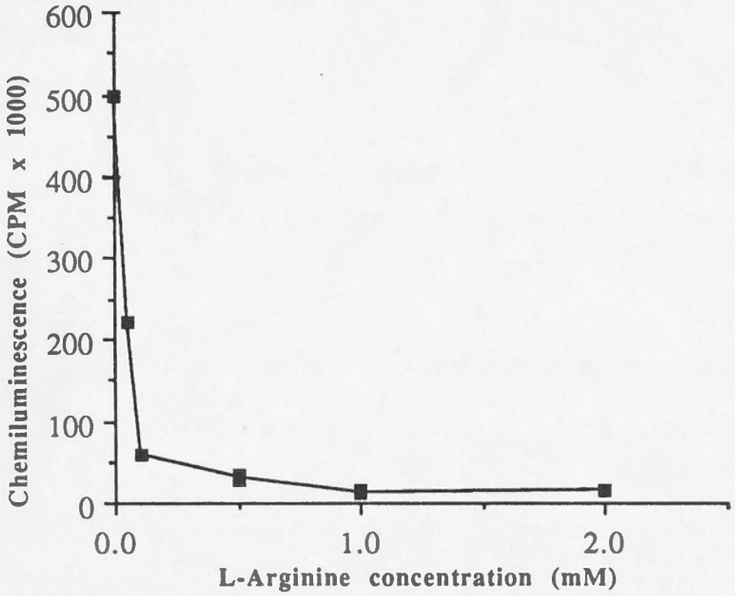
Fig 3.7: Peak CL response to PMA with increasing L-arginine concentration in human PMNs.

5×10^5 cells were used and the experimental conditions were the same as those described for HL60 and 9 day DMSO-treated cells. All data are the means $\pm$ S.E.M. of at least three separate experiments, each performed in duplicate.

Fig 3.8: Enhancement of the peak CL response to PMA in the presence of L-arginine (0.1mM) with increasing L-NMMA concentration in human PMNs.

5×10^5 cells were used and the experimental conditions were the same as those described for HL60 and 9 day DMSO-treated cells. All data are the means $\pm$ S.E.M. of at least three separate experiments, each performed in duplicate. The CL response in the presence of 0.02 and 0.05mM L-NMMA is significantly greater than that in the absence of L-NMMA, ($p<0.01$).

(3.7) Peak CL response to PMA with increasing L-arginine concentration in human PMNs



(3.8) Enhancement of peak CL response to PMA in the presence of L-arginine (0.1mM) with increasing L-NMMA concentration in human PMNs

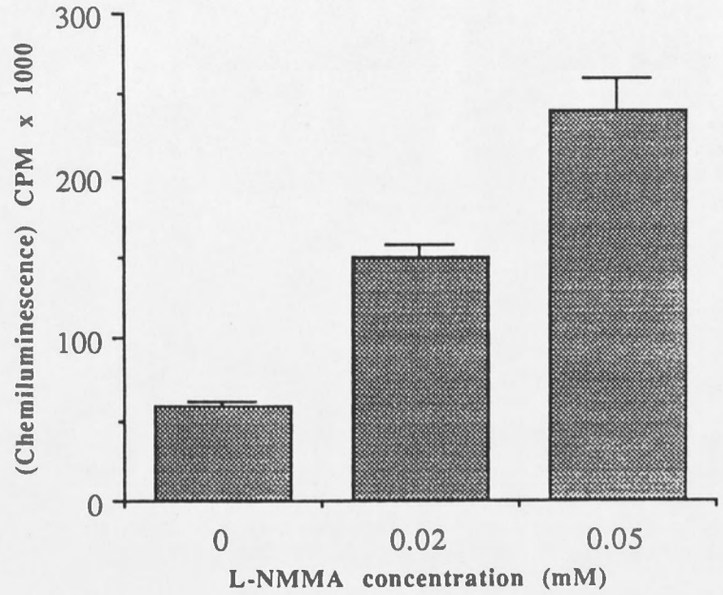


Fig 3.9: Effect of increasing concentration of L-NMMA on the peak CL response in the absence of added L-arginine in human PMNs.

5×10^5 cells were used and the experimental conditions were the same as described for HL60 and 9 day DMSO-treated cells. All data are the means $\pm$ S.E.M. of at least three separate experiments, each performed in duplicate. The CL response in the presence of 0.5, 1.0 and 5mM L-NMMA is not significantly different from that in the absence of L-NMMA, ($p>0.1$).

(3.9) Effect of increasing L-NMMA concentration on the peak CL response in the absence of added L-arginine in human PMNs

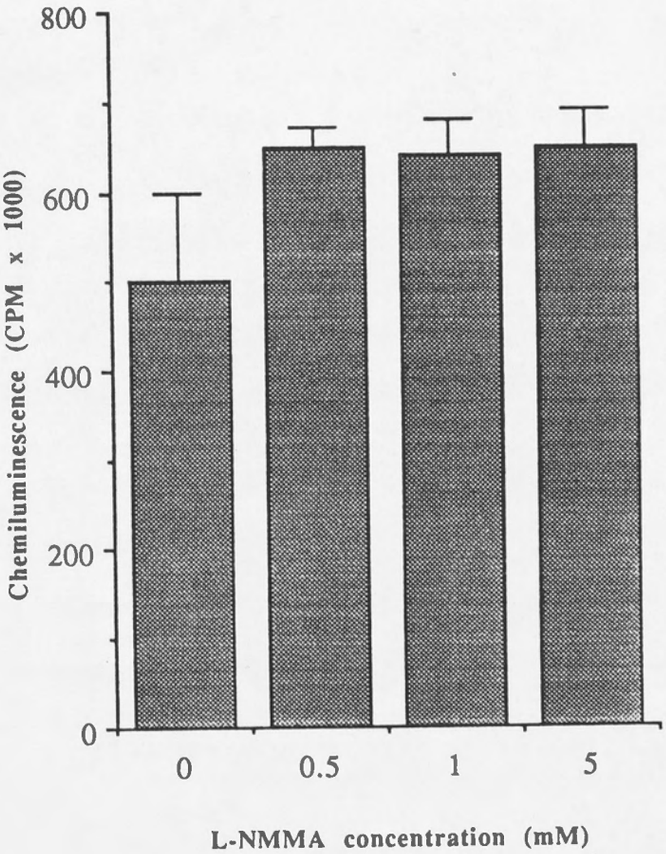


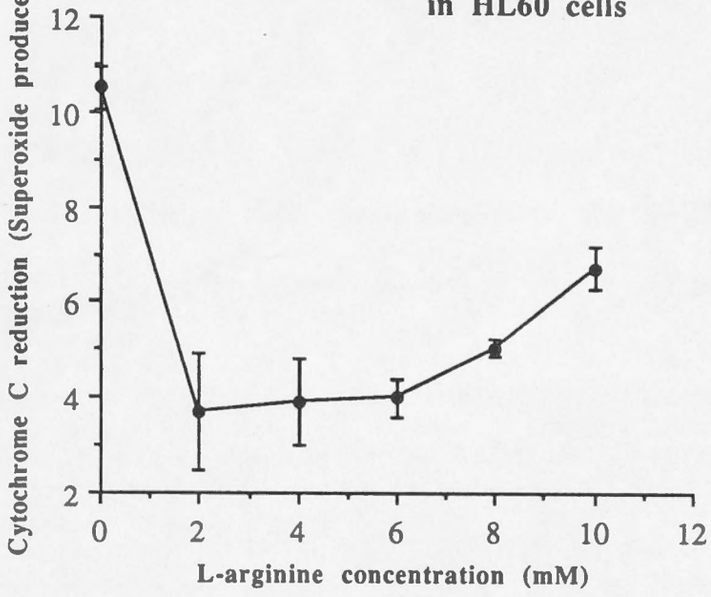
Fig 3.10: The inhibition of PMA-stimulated superoxide production in the presence of increasing concentration of L-arginine in HL60 cells.

The conditions for the experiment are described in the Materials and Methods section. Superoxide produced in 10 mins is expressed as nmole/2 x 10^6 cells. All data are the means $\pm$ S.E.M. of at least three separate experiments, each performed in duplicate.

Fig 3.11: Enhancement of PMA-stimulated superoxide production with increasing L-NMMA concentration at 10mM L-arginine in HL60 cells.

The conditions for the experiment are described in the Materials and Methods section. Superoxide produced in 10 mins is expressed as nmole/2 x 10^6 cells. All data are the means $\pm$ S.E.M. of at least three separate experiments, each performed in duplicate. Superoxide produced in the presence of 1, 5 and 10mM L-NMMA is significantly greater than that in the absence of L-NMMA, ($p<0.02$).

(3.10) Inhibition of PMA-stimulated superoxide production in the presence of increasing concentration of L-arginine in HL60 cells



(3.11) Enhancement of PMA-stimulated superoxide production with increasing L-NMMA concentration at 10 mM L-arginine in HL60 cells

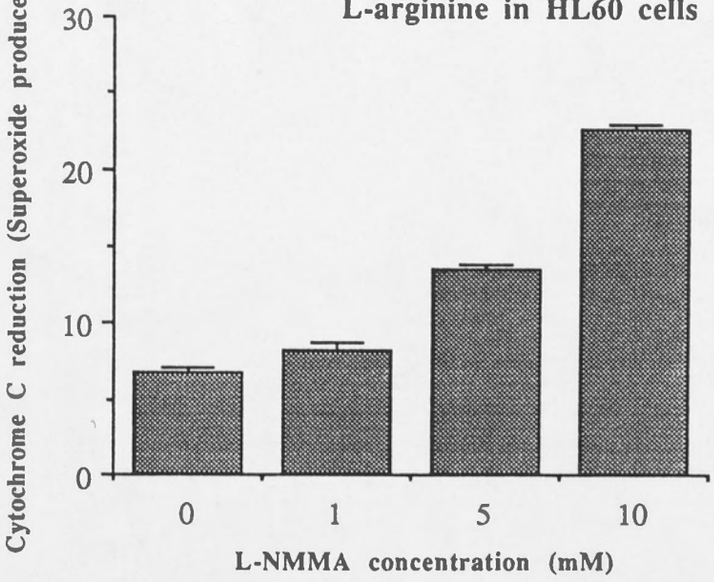


Fig 3.12: Enhancement of PMA-stimulated superoxide production with increasing concentration of L-NMMA in the absence of L-arginine in HL60 cells.

The conditions for the experiment are described in the Materials and Methods section. Superoxide produced in 10 mins is expressed as nmole/2 x 10^6 cells. All data are the means $\pm$ S.E.M. of at least three separate experiments, each performed in duplicate. Superoxide produced in the presence of 1, 5 and 10mM L-NMMA is significantly greater than that in the absence of L-NMMA, ($p<0.05$).

(3.12) Enhancement of PMA-stimulated superoxide production with increasing concentration of L-NMMA in HL60 cells

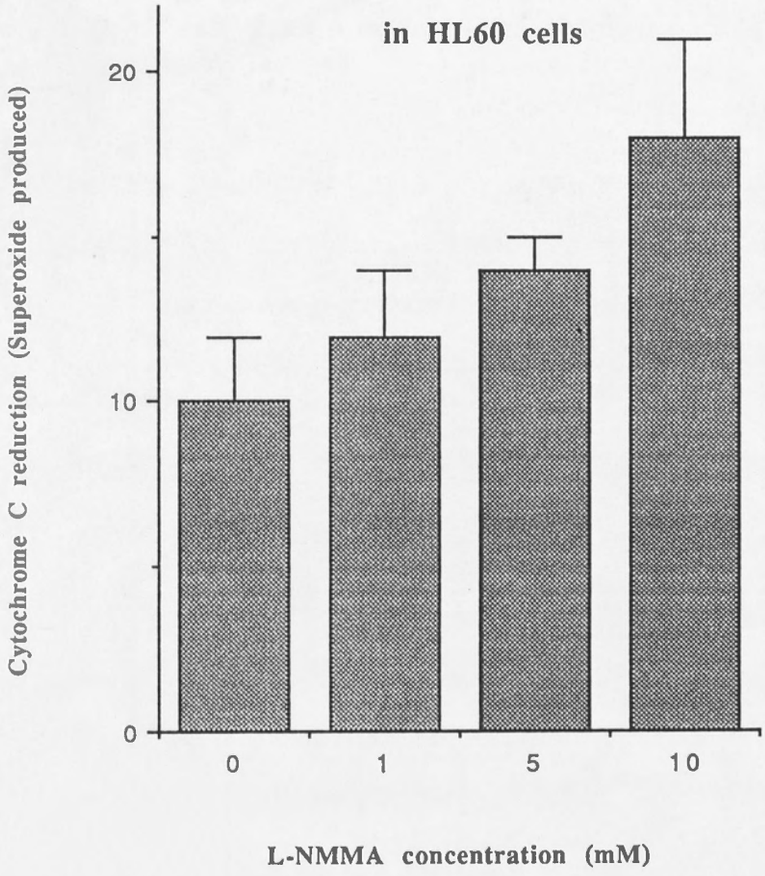


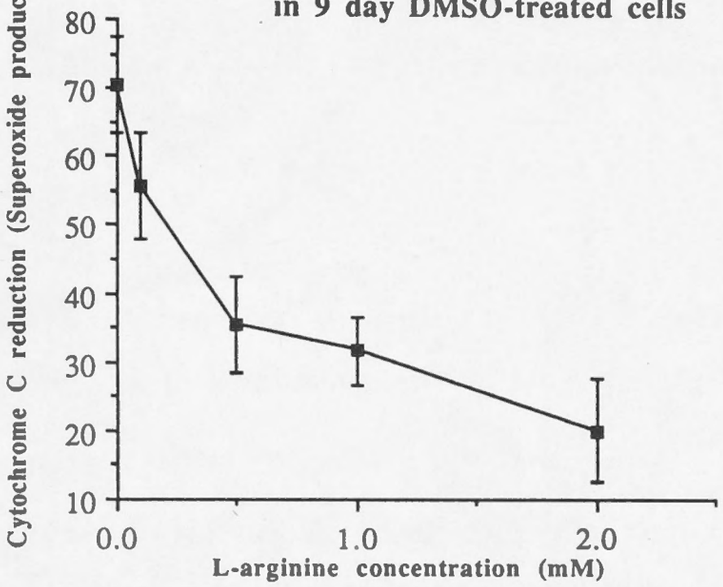
Fig 3.13: Inhibition of PMA-stimulated superoxide production in the presence of increasing L-arginine concentration in 9 day DMSO-treated cells.

The conditions for the experiment are described in the Materials and Methods section. Superoxide produced in 10 mins is expressed as nmole/2 x 10^6 cells. All data are the means ± S.E.M. of at least three separate experiments, each performed in duplicate.

Fig 3.14: Enhancement of PMA-stimulated superoxide production with increasing L-NMMA concentration at 1mM L-arginine in 9 day DMSO-treated cells.

The conditions for the experiment are described in the Materials and Methods section. Superoxide produced in 10 mins is expressed as nmole/2 x 10^6 cells. All data are the means ± S.E.M. of at least three separate experiments, each performed in duplicate. Superoxide produced in the presence of 0.4, 0.8 and 1mM L-NMMA is significantly greater than that in the absence of L-NMMA, ($p<0.05$).

(3.13) Inhibition of PMA–stimulated superoxide production in the presence of increasing L-arginine concentration in 9 day DMSO-treated cells



(3.14) Enhancement of PMA–stimulated superoxide production with increasing L-NMMA concentration at 1mM L-arginine in 9 day DMSO-treated cells

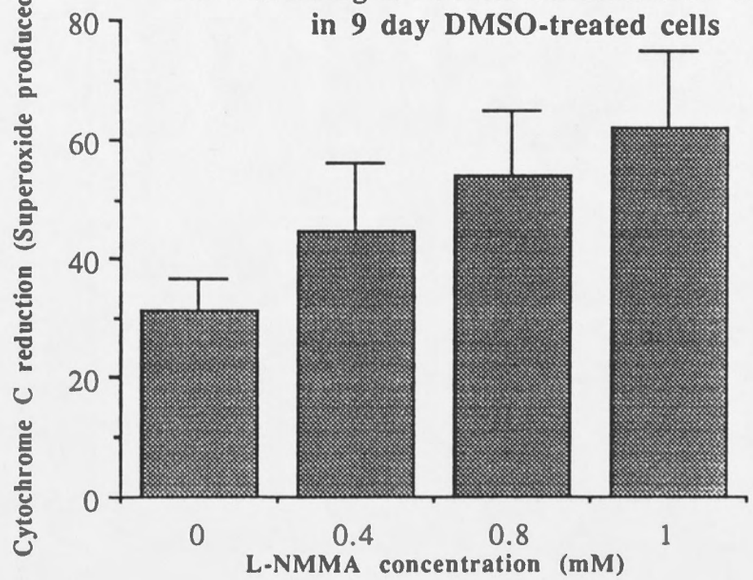


Fig 3.15: Enhancement of PMA-stimulated superoxide production with increasing L-NMMA concentration in the absence of L-arginine in 9 day DMSO-treated cells.

The conditions for the experiment are described in the Materials and Methods section. Superoxide produced in 10 mins is expressed as nmole/2 x 10^6 cells. All data are the means $\pm$ S.E.M. of at least three separate experiments, each performed in duplicate. Superoxide produced in the presence of 1,.4, and 8mM L-NMMA is significantly greater than that in the absence of L-NMMA, ($p<0.04$).

(3.15) Enhancement of PMA-stimulated superoxide production with increasing L-NMMA concentration in 9 day DMSO-treated cells

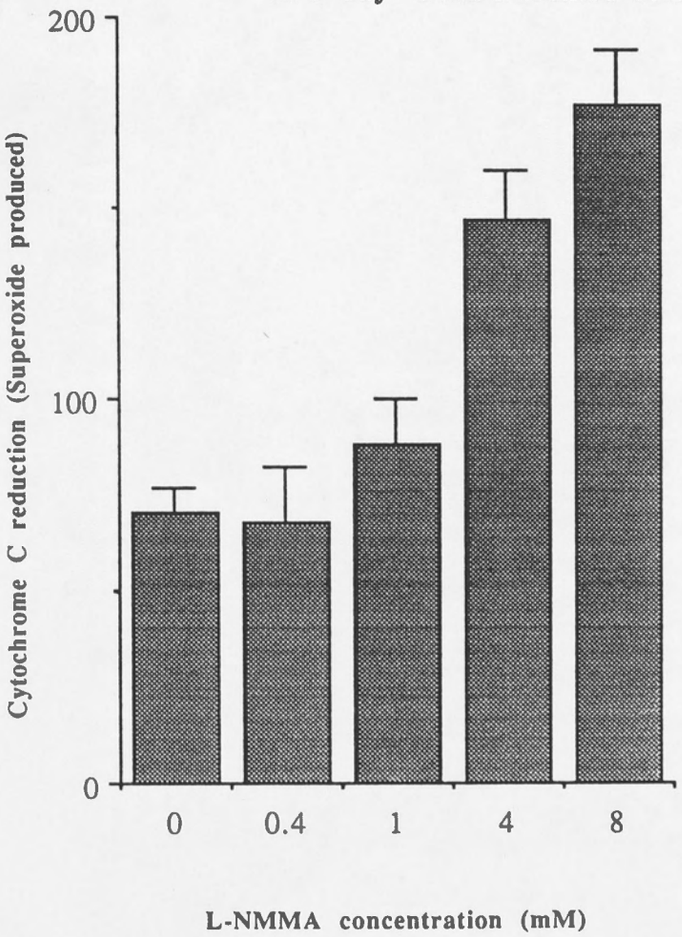
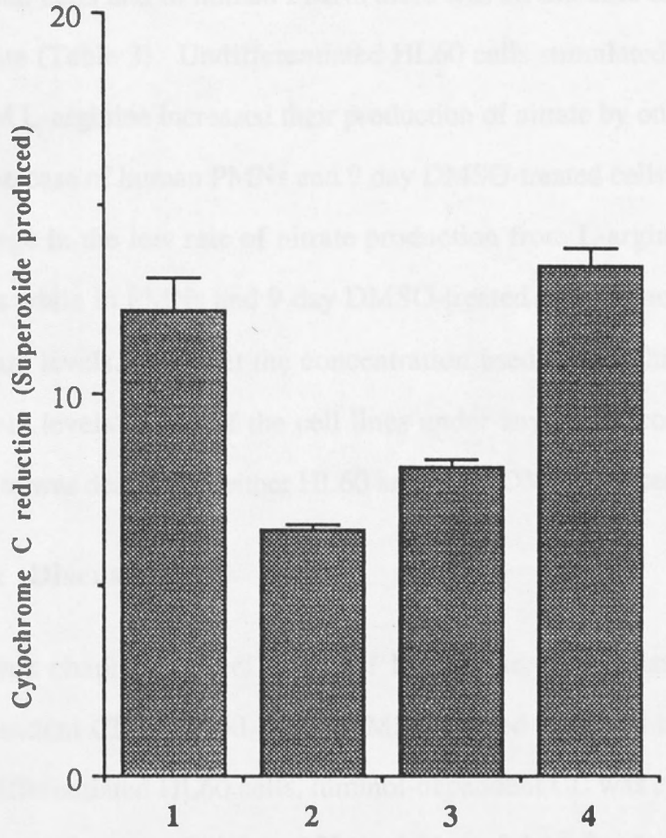


Fig 3.16: PMA-stimulated superoxide production in human PMNs.

(1) PMNs only; (2) (+) 1mM L-arginine; (3) (+)1mM L-arginine and 0.5mM L-NMMA; (4) (+) 1mM L-NMMA only. 5 x 10^5 cells were used and superoxide produced in 10 mins is expressed as nmole/5 x 10^5 cells. The rest of the conditions were the same as those described for HL60 and 9 day DMSO-treated cells. All data are the means $\pm$ S.E.M. of at least three separate experiments, each performed in duplicate. (2) is significantly smaller than (1) ($p< 0.01$), (3) is significantly greater than (2) ($p< 0.01$), and (4) is significantly greater than (1) ($p< 0.03$).

(3.16) PMA-stimulated superoxide production under different conditions in human PMNs



3.2.3 Estimation of nitrate and nitrite

The basal levels of nitrate detected in HL60 and 9 day DMSO-treated cells were approximately the same (Table 3), whilst in freshly-isolated human PMNs this level was approximately eight-fold higher. Stimulation of undifferentiated HL60 cells with PMA in the absence of L-arginine caused no change in the level of nitrate, while in 9 day DMSO-treated cells and in human PMNs there was an increase of 22-25% in the production of nitrate (Table 3). Undifferentiated HL60 cells stimulated with PMA in the presence of 2mM L-arginine increased their production of nitrate by only 6%, compared with 32-40% in the case of human PMNs and 9 day DMSO-treated cells. L-NMMA (3mM) caused no change in the low rate of nitrate production from L-arginine by undifferentiated HL60 cells while in PMNs and 9 day DMSO-treated cells it caused a reduction of 20% in the nitrate levels. SOD at the concentration used (120U) had no significant effect on the nitrate levels in any of the cell lines under any of the conditions tested (Table 3). No nitrite was detected in either HL60 and 9 day DMSO-treated cells.

3.3: Discussion

In this chapter a novel effect of L-arginine metabolism on PMA-induced luminol-dependent CL in HL60, 9 day DMSO-treated cells and human PMNs is described. In undifferentiated HL60 cells, luminol-dependent CL was *enhanced* markedly in response to increasing concentrations of L-arginine and the enhancement was inhibited significantly by L-NMMA (Figs 3.1, 3.2), a competitive inhibitor of L-arginine metabolism by nitric oxide synthase. L-NMMA in the presence of L-arginine inhibited the enhanced luminol CL in a concentration-dependent manner (Fig 3.2), but L-NMMA by itself, in the absence of L-arginine, had no effect on the luminol CL response (Fig 3.3). This indicates that a large part of the luminol-dependent CL response measured in PMA-activated HL60 cells in the absence of L-arginine is dependent on the catalytic activity of NADPH-oxidase rather than on nitric oxide synthase. Luminol-amplified CL in a cell system is dependent primarily on the production of ROI, which might occur by direct

Table 3 : Nitrate production by HL60, 9 day DMSO-treated cells and human PMNs.

Cell Type	Basal level of nitrate in the absence of L-arginine (µmole/10^6cells)	Nitrate formed in the absence of L-arginine (µmole/10^6cells)	Nitrate formed in the presence of L-arginine (2mM) (µmole/10^6cells)	Nitrate formed in the presence of L-arginine (2mM) + L-NMMA (3mM) (µmole/10^6cells)	Nitrate formed in the presence of L-arginine (2mM) + SOD (120U) (µmole/10^6cells)
Undifferentiated HL60 cells	0.49±0.01	0.52±0.00	0.53±0.01	0.52±0.02	0.53±0.01
9 day DMSO-treated cells	0.40±0.00	0.52±0.01	0.67±0.15[a]	0.54±0.05[c]	0.59±0.12
PMNs	3.39±0.08	4.54±0.09	4.94±0.19[b]	3.95±0.11[d]	5.20±0.04

Experimental conditions are described in the Materials and Methods section. The basal level of nitrate was measured without stimulation of cells with PMA. Under all of the other conditions cells were stimulated with PMA (1.5µg/ml). All data are the means ± S.E.M. of three separate experiments, each performed in duplicate. a and c are significantly different from HL60 cells ($p<0.05$); b and d are significantly different from HL60 cells ($p<0.01$).

activation of the NADPH-oxidase system or, possibly, by the coupling of superoxide anion production with the formation of the nitric oxide radical, leading eventually to the formation of a diamagnetic peroxynitrite anion ($ONOO^-$) which is known to decompose to strong oxidants ($OH^{\cdot}$) and the nitrogen dioxide radical ($NO_2^{\cdot}$) en route to nitrate (Beckman *et al.*, 1990) (Scheme 3).

$$O_2^{-\cdot} + NO^{\cdot} \rightarrow ONOO^- + H^+ \rightarrow ONOOH \rightarrow OH^{\cdot} + NO_2^{\cdot} \rightarrow NO_3^- + H^+$$

Oxidants such as ($OH^{\cdot}$) and the nitrogen dioxide radical ($NO_2^{\cdot}$) may be responsible, by some presently unknown mechanism, for the amplification of the luminol-dependent CL signal. Luminol CL in cell systems is thought to be largely dependent on the co-oxidation of luminol by H_2O_2 and HOCl (Brestel, 1985). The possibility that H_2O_2 participates as a second reactant with nitric oxide in the sequential oxidation of luminol can also be proposed. In undifferentiated cells, an enhancement in the CL response due to L-arginine could be seen after a lag phase of approximately 5 mins. The peak response occurred at 18-20 mins, by which time the peak CL due to ROI production was almost over and only a very low rate of ROI production remained. That the elevation of the delayed CL response in the presence of L-arginine occurred as a result of the interaction of oxygen radicals with nitric oxide is indicated by the inhibition of this response by L-NMMA (Fig 3.2). In some cases, higher concentrations of L-NMMA inhibited not only the L-arginine enhanced response, but also a proportion of the response produced by oxygen radicals via NADPH-oxidase (Fig 3.2), indicating that nitric oxide may play some part in the production of ROI. These results are consistent with the inhibition of O_2^- production, measured by the reduction of cytochrome C, in the presence of L-arginine (Fig 3.10). O_2^- production in the presence of L-arginine was relieved by the addition of L-NMMA to the incubation medium (Fig 3.11), which is indicative of inactivation (or removal) of O_2^- by nitric oxide (McCall *et al.*, 1989). L-NMMA in the absence of L-arginine actually enhanced the production of O_2^-, suggesting that there may normally be some inactivation of NADPH-oxidase products by the basal levels of nitric oxide present (Fig 3.12). However, the fact that L-NMMA by itself, in the absence of L-arginine, had no effect on

the luminol-dependent CL responses (Fig 3.3) is consistent with the idea that O_2^- does not normally play a direct role in the co-oxidation of luminol by the H_2O_2/HOCl system that gives rise to CL (Brestel, 1985). The lack of a Mn^{+2}-dependent SOD in HL60 cells (Oberley and Buettner, 1979) may contribute to the failure of formation of H_2O_2 (and hence HOCl) in the presence of L-arginine, maintaining luminol CL (which would then be dependent on the spontaneous dismutation of O_2^- to H_2O_2) at a static level. The activity of peptidylarginine deiminase, recently discovered in murine bone marrow cells, which can transform the arginine residues of proteins into citrulline and possibly nitric oxide (Kamoun *et al.*, 1988), may also be responsible for the enhancement of O_2^- production by L-NMMA. This enzyme is superoxide-dependent and causes the production of citrulline even in the absence of added L-arginine. The activity of peptidyl arginine deiminase may play an important role in sustaining the concentration of nitric oxide at a basal level necessary for the maintenance of normal physiological functions of the cells. In addition to its possible role in the production of nitric oxide, this enzyme is also important for the synthesis of citrulline, a precursor molecule for polyamine synthesis (via ornithine and the urea cycle) that is associated with normal cell proliferation (Luk *et al.*, 1982; Suguira *et al.*, 1984).

Very low concentrations of endogenous nitrate, possibly derived from nitric oxide or some unknown unoxidized nitrogenous radicals (Mitchell *et al.*, 1916 ; Green *et al.*, 1981), were measured in undifferentiated HL60 cells (Table 3). Nitric oxide was concluded to be a likely source of this nitrate because of the ability of PMA to stimulate the production of nitrate (although only by 6%). The failure of L-NMMA to decrease the nitrate level may not mean that undifferentiated HL60 cells fail to produce nitric oxide in the presence of L-arginine (which would be contrary to the results of the CL response and cytochrome C reduction) but that these cells produce nitrate in response to PMA at such low levels that the effect of L-NMMA at low concentrations (3mM) is not easily measurable by the nitrate electrode.

In 9 day DMSO-treated cells, the actions of L-arginine and L-NMMA had very different effects on luminol-dependent CL, while the effects on O_2^- formation remained the same as those in undifferentiated HL60 cells. In these cells, L-arginine *inhibited* the CL response in a concentration-dependent manner (Fig 3.4). L-NMMA relieved this inhibition by enhancing the response in the presence of L-arginine in a dose-dependent manner (Fig 3.5). The inhibition of the luminol-dependent CL response by L-arginine in 9 day DMSO-treated cells occurred possibly because of the inactivation or removal of O_2^- by nitric oxide. In these cells, kinetically overlapping luminol CL responses due to the simultaneous formation of ROI and nitric oxide in the presence of L-arginine were observed. The formation of the peroxynitrite anion ($ONOO^-$) is dependent on the presence of a saturating concentration of nitric oxide in relation to that of O_2^-(Blough and Zafiriou, 1985); at nitric oxide concentrations equal to or lower than that of O_2^-, peroxynitrite anion is not formed (Blough and Zafiriou, 1985). Schmidt *et al.* (1988) reported that three orders of magnitude more O_2^- than nitric oxide was produced when dibutyryl-cAMP-differentiated HL60 cells and human PMNs were activated with a chemotactic peptide. If a similar situation exists in 9 day DMSO-treated cells, peroxynitrite anion ($ONOO^-$) and related oxidants (such as $OH^\cdot$ and $NO_2^\cdot$) that might elevate the CL response in HL60 cells, would not be formed. L-arginine inhibited the formation of O_2^- in a concentration-dependent manner in DMSO-treated cells (Fig 3.13) and, at the same time, increased the production of nitric oxide as measured by the appearance of nitrate (Table 3). The inhibition of O_2^- production at 2mM L-arginine (50%, Fig 3.13) is proportional to the enhancement of the nitrate level (40%, Table 3) at the same L-arginine concentration. This might indicate that nitric oxide is responsible for scavenging reactive oxygen species (O_2^-) or that the NADPH-oxidase pathway is inhibited due to the competition for NADPH as the L-arginine-dependent nitric oxide synthesis pathway becomes active. L-NMMA, in the presence and absence of L-arginine, had a stimulatory effect on O_2^- production (Figs 3.14 and 3,15), again consistent with a scavenging action of basal nitric oxide for superoxide anion (O_2^-). However, when L-NMMA was present on its own, it paradoxically inhibited luminol-

dependent CL responses in 9 day DMSO-treated cells (Fig 3.6), suggesting that nitric oxide may be more directly involved in the co-oxidation of luminol by H_2O_2/HOCl (Brestel, 1985). An interplay of nitric oxide and O_2^-/H_2O_2 in producing luminol-dependent CL has been suggested by cell-free experiments with authentic nitric oxide and the O_2^-/H_2O_2 generating system xanthine/xanthine oxidase (Wang *et al.*, 1991). These authors have shown that luminol-dependent CL was only emitted when both nitric oxide and O_2^-/H_2O_2 were present simultaneously. The exact chemical species that give rise to this type of CL was not explained however.

In 9 day DMSO-treated cells, the basal level of nitrate present was the same as that in undifferentiated HL60 cells (Table 3). Stimulation by PMA, even in the absence of L-arginine, led to a 22% increase in the level of nitrate, which may have come from endogenous L-arginine as reported for other cells (Iyengar *et al.*, 1987). In the presence of 2mM L-arginine, PMA stimulation of 9 day DMSO-treated cells led to a 40% increase in the nitrate levels, only 20% of which was inhibited by 3mM L-NMMA. The implied increased synthesis of nitric oxide in 9 day DMSO-treated cells compared to the untreated HL60 cells may be associated with growth inhibition in these terminally-differentiated cells. Elevated levels of nitric oxide have been reported to inhibit mitochondrial respiration, the citric acid cycle enzyme aconitase (Hibbs *et al.*, 1987) and DNA synthesis (Lepoivre *et al.*, 1991) by disrupting enzymes containing iron-sulphur clusters in tumour cells. Ribonucleotide reductase is a key enzyme involved in DNA synthesis that is also a target site for the cytotoxic action of nitric oxide (Lepoivre *et al.*, 1990). Nitric oxide interacts with a tyrosyl radical present in the M_2-subunit of the enzyme. This subunit contains iron involved in the stabilization of the tyrosyl radical. The loss of the radical could be due to the nitric oxide-dependent mobilization of iron (Lepoivre *et al.*, 1991).

Human PMNs, when activated by PMA, behaved like 9 day DMSO-treated cells with respect to luminol-dependent CL, cytochrome C reduction and nitrate production (Figs 3.7, 3.8 , 3.16). The only difference between authentic PMNs and DMSO-treated cells was the lack of of enhancement of the CL response by L-NMMA in the absence of L-

arginine. The discrepancy in the behaviour of human PMNs and DMSO-treated cells might be due to the eight-fold difference in the basal levels of nitrate that may be related, in turn, to a higher rate of endogenous nitric oxide formation in PMNs (Table 3). In the absence of L-arginine, L-NMMA inhibited basal nitric oxide formation in the human PMNs, thereby relieving the scavenging action of nitric oxide for O_2^-. As a result the rate of O_2^- formation was enhanced (Fig 3.16), which would have caused a rise in H_2O_2/HOCl production responsible for the enhanced luminol CL response. On the other hand, in DMSO-treated cells , L-NMMA caused an inhibition of the luminol response even in the presence of increased production of O_2^-, possibly because of decreased H_2O_2/HOCl due to the virtual absence of myeloperoxidase (Chapter 2, Section 2.19). The elevated levels of nitrate in fully differentiated human PMNs may be associated with the deployment of nitric oxide as a cytotoxic metabolite, as has been demonstrated for human macrophages (Billiar *et al.*, 1989). DMSO-treated cells failed to exhibit such high levels of endogenous nitrate, which may be associated with their less effective cytotoxic armoury compared with human PMNs (Chapter 2, Section 2.19). Although the catabolism of L-arginine via the nitric oxide pathway can promote cytotoxicity, the concomitant metabolic inhibition may lead to the suppression of PMN function as shown by the decreased production of O_2^- when nitric oxide synthesis is operative. Similar findings have been reported for activated macrophages (Albina *et al.*, 1989) and it may represent, in part, a cytoprotective function towards target cells conferred by nitric oxide (Rubanyi *et al.*, 1991).

Nitric oxide mediates its functions in different ways in different cell lines and tissues. Although the exact function and mechanism of action of nitric oxide is still under investigation, some of its functions in phagocytic cells (e.g. macrophages), endothelial cells and brain cells have been defined, and the possible involvement of intracellular cGMP (via guanylate cyclase) as a second messenger has been suggested (Moncada and Higgs, 1991). The function of nitric oxide in tumour cells is not clear, although minute amounts may be necessary to sustain cell proliferation. The mechanism by which nitric

oxide might sustain proliferation is not known, but the involvement of cGMP as a second messenger has been suggested (Efron *et al.*, 1991). In contrast, the inhibition of proliferation induced by nitric oxide in other cell types (Albina *et al.*, 1991; Garg and Hassid, 1990; Lepoivre *et al.*, 1991) is thought to be a cGMP-independent process, and the possible involvement of ADP-ribosylation has been suggested (Garg and Hassid, 1990). NAD-dependent ADP-ribosylation and adenylate cyclase activation have been proposed as regulatory mechanisms involved in the anti-tumour activity of L-arginine (Takeda *et al.*, 1975; Chany and Cerutti, 1982; Barbul, 1986; Cho-Chung *et al.*, 1980). The possibility also exists that the modification of hormonal changes in the host induced by dietary supplementation with L-arginine may also cause decreased tumourigenicity (Barbul, 1986).

The inhibition of O_2^- production by L-arginine in HL60, DMSO-treated cells and human PMNs due to the scavenging and cytoprotective actions of nitric oxide was reported in human PMNs during the progress of the present work (Rubanyi *et al.*, 1991; McCall *et al.*, 1989; Wright *et al.*, 1989). However, the current work is the first report in which the enhancement and/or inhibition of luminol-dependent CL by L-arginine has been demonstrated in HL60, DMSO-treated cells and human PMNs. A possible role for peroxynitrite ($ONOO^-$) in the enhancement and inhibition of luminol CL in the three different cell types has been suggested. In HL60 cells, the peroxynitrite anion ($ONOO^-$) possibly starts forming after a lag phase of approximately 5 mins when the levels of O_2^- have fallen and the concentration of nitric oxide synthesized by L-arginine exceeds that of O_2^-. The formation of peroxynitrite anion ($ONOO^-$) may lead to the production of strong oxidants with the capacity to elevate the luminol response. On the other hand, in DMSO-treated cells and human PMNs, the high rate of O_2^- production in response to PMA (even though it is inhibited somewhat by L-arginine) maintains a large excess of ROI in relation to that of nitric oxide, leading to inhibited formation of the peroxynitrite anion ($ONOO^-$). Coupled to this phenomenon is the inhibition of O_2^- formation by L-arginine which curtails the luminol-dependent CL responses drastically. It is proposed as a unifying idea

that the formation of precursor components of peroxynitrite anion formation may be responsible for the enhancement of the luminol-dependent response in cells such as HL60's, with low capacity for ROI production, while in cells which produce elevated levels of ROI the formation of the peroxynitrite anion is inhibited. The enhancement of the luminol CL response has been observed in isolated hepatocytes (Wang *et al.*, 1991), which presumably produce ROI in much smaller amounts than PMNs, and a possible role for peroxynitrite has been suggested in relation to the elevation of this response.

In spite of small differences, 9 day DMSO-treated cells behave like human PMNs with respect to RNI formation. Undifferentiated HL60 cells, on the other hand, show a marked difference in RNI production, especially in terms of their luminol-dependent CL response to PMA. The difference between the cells may be due to the different modes of regulation of RNI formation, which may be related, in turn, to differences in the physiological effects of this labile, vasoactive molecule in the three cell systems.

Chapter 4: Tumourigenicity/leukaemogenicity induced in nude mice by leukaemic and chemically-differentiated HL60 cells

4 Introduction

Chapters 2 and 3 have demonstrated the usefulness of cultured HL60 cells for the analysis of leukaemic cell differentiation *in vitro*. However, for studies on cell proliferation and differentiation *in vivo*, animal models in which leukaemic cells can be grown and maintained have been sought (Potter *et al.*, 1985). In any study of the leukaemic HL60 cell line – with its unique bipotential capability of differentiating either to a granulocytic or monocytic end point (depending on the inducer used) – it is important to verify the extent to which these cells have differentiated in response to the inducers used by using an animal model to confirm whether there is a corresponding change in tumourigenicity. Reports on mouse myeloid leukaemic cells (M_1) have shown that no leukaemogenicity develops in SL mice when these cells are differentiated *in vitro* and transplanted intraperitoneally in diffusion chambers (Honma *et al.*, 1978). The degree of leukaemogenicity was measured in this case by recording the survival time of mice with leukaemia. In a similar way, macrophage-like cells that develop spontaneously from the leukaemic M_1 cell line also lose their ability to induce leukaemia in animals (Maeda and Ichikawa, 1973). Several clones of inducer-resistant M_1 cells, which do not differentiate *in vitro* even when treated with high concentrations of the inducer, have been isolated from the parent cell line. These cells have been a valuable tool for use in studies on the relationship between leukaemogenicity/tumourigenicity of cells and the inducibility of their differentiation. Sensitive M_1 cells that had been treated with the inducer *in vitro* showed no leukaemogenicity in mice whereas, after similar treatment, inducer-resistant cells failed to show any decrease in leukaemogenicity *in vivo* (Ichikawa, 1970; Fibach and Sachs, 1974). These findings suggest that the differentiation of leukaemic cells may be associated with a loss of leukaemogenicity. Inducers of cell differentiation significantly enhance the survival time of mice transplanted with inducer-sensitive cells but scarcely affect the survival time of mice injected with resistant cells (Honma *et al.*, 1979; Potter *et al.*, 1985), suggesting that there is an association between the effects of inducers in stimulating cell differentiation *in vivo* and the increased survival time of the mice. The studies described so far were carried out on syngeneic or allogeneic inbred

mice. When immunodeficient nude mice were used as an experimental model for the maintenance of leukaemic cells, however , these cells failed to develop leukaemia in animals but instead produced a solid sarcoma at the site of inoculation. This has been demonstrated in the case of leukaemic HL60 and K562 cells (Potter *et al.*, 1985; Machado *et al.*, 1977).

Aim of the study

This chapter describes tumourigenicity/leukaemogenicity associated with the growth of undifferentiated and differentiated HL60 cells. The extent to which tumourigenicity/leukaemogenicity decreased when these cells were differentiated *in vitro* by DMSO, butyrate and PMA is also discussed. This was done by injecting undifferentiated and differentiated HL60 cells to immunosuppressed, cyclophosphamide primed nude mice (Section 4.1.3). In order to assess the degree of tumourogenicity of the tumour cells, in some cases, cells collected from the tumours were injected back to the mice. Leukemogenicity in the mice was detected by doing differential blood count, haematocrit value and haemoglobin estimation of the tumour-bearing and non-tumour bearing mice (Section 4.1.5). This study was important in making an assessment of the differentiating capabilities of the inducers used (DMSO, butyrate and PMA), and in exploring the possible relationship between tumourigenicity/leukaemogenicity and the inducibility of differentiation of HL60 cells *in vitro*. This chapter also characterizes the tumour cells retrieved from the nude mice in order to clarify their relationship with the parental cells responsible for the development of the tumours. For this purpose experiments were designed to investigate the cytology and phenotypic characteristics of the tumour cells before and after differentiation. This was done by putting the tumour cells in culture and then differentiaing them by DMSO, butyrate and PMA. The degree of differentiation in both undifferentiate and differentiated tumour cells was assessed by morphological examination (Section 4.1.4), by the oxidative and non-oxidative activities which were done by estimating the level of ROI production (by luminol-dependent CL and cytochrome C reduction) and secretion of lysosomal enzymes (Section 4.1.6). In

each case undifferentiated and the respective differentiated cultured cells served as control.

4.1 Materials and Methods

4.1.1 Chemicals

Cyclophosphamide was obtained from Sigma Chemical Company (U.S.A) and the remaining analytical grade chemicals were obtained from the sources described in Chapter 2, Section 2.1.1.

4.1.2 Cells

HL60 cells were maintained and induced to differentiate by DMSO, PMA and butyrate as discussed in Chapter 2, Sections 2.1.2 and 2.1.3.

4.1.3 Animals

Six weeks old athymic male nude mice (Swiss nu/nu) were supplied by the Animal Breeding Establishment, John Curtin School of Medical Research, The Australian National University. The mice were bred in a specific-pathogen-free unit, from hairy females and nude male mice, which produced litters of both populations in a 1:1 ratio. The nude mice used in the experiment were housed (not more than five per cage) under specific pathogen-free conditions, using clean racks. The animals were kept in a room maintained at 22 ± 2^{o} C and 60-65% humidity. Mice were allowed access to water and laboratory chow *ad libitum* (Rural Stock Feed, Hall, A.C.T, Australia). Approximately 3×10^{6}-4×10^{7} cells in RPMI-1640 medium (0.2ml and free of serum) were injected into mice, subcutaneously or intraperitoneally, under aseptic conditions, using a 19 gauge needle. Eighty five mice were used overall, five in each group. In the first group (5 in each group), undifferentiated and differentiated HL60 cells (differentiated with either DMSO or butyrate or PMA) were transplanted, subcutaneously or intraperitoneally, 3 days after a priming dose of cyclophosphamide (250mg). The second group of mice (5 mice), which served as controls, was injected with RPMI-1640 medium alone (0.2ml,

serum free) 3 days after priming with cyclophosphamide. The third control group (5 mice) received injections of RPMI-1640 medium alone (0.2ml, serum free) on both days; while the fourth group (5 mice per group) was injected with undifferentiated cells (3 x 10^6) either subcutaneously or intraperitoneally 3 days after injection of RPMI-1640. Successfully-differentiated cells were identified before transplantation from the rate of ROI formation after stimulation with PMA as described in Section 2.1.3. Injected mice were observed at 3 day intervals and the rate of tumour growth was estimated on the basis of tumour volume. This was calculated from the external measurement of tumour dimension with a plastic tape measure and fitted to the following equation (Machado *et al.*, 1977):

$$\text{Volume (cm}^3\text{)} = \pi/12 \times (a^2b)$$

where: a = major diameter (cm)

b = minor diameter (cm)

Because of the asymmetrical nature of the tumours this formula gives an accurate and consistent measure of tumour volumes as it takes into account both the major and minor diameters.

4.1.4 Cytological studies

Using aseptic technique, tumours that developed in nude mice at various intervals were excised after killing the mice by exposure to an atmosphere of 100% CO_2. The tumours were washed in culture medium (RPMI 1640), weighed, cut into several small pieces and teased mechanically to produce individual cells. Cell viability was checked by trypan blue exclusion as described in Section 2.1.2. Tumour cells were put into culture, maintained under the same conditions as HL60 cells (Section 2.1.2) and induced to differentiate (Section 2.1.3) after they had been in culture for at least three weeks. The morphological and phenotypic characteristics of the undifferentiated and differentiated tumour cells were assessed as described in Section 2.1. In some cases the tumour cells were re-transplanted both subcutaneously and intraperitoneally (3 x 10^6 cells) into nude mice and the rate of tumour development was observed a second time as described above.

4.1.5 Studies on mice

Blood from both tumour-bearing and non-tumour bearing mice was collected by cutting the edge of their tails. Differential cell counts, the haematocrit value and the haemoglobin content of the blood obtained from the tails of the mice were performed as follows:

4.1.5 (a) Differential count

The differential white cell count of the blood of both the tumour-bearing and non-tumour-bearing nude mice was performed by light microscopy using oil at x 1000 magnification on smears stained with Wright-Giemsa Stain (BDH chemicals, Australia Pty Ltd). Approximately 100 cells were counted in different visual fields and the percentages of white blood cells and reticulocytes were determined.

4.1.5 (b) Haematocrit value

The haematocrit value or the packed cell volume was determined using micro-capillary tubes of 30 mm in length and 1mm in internal diameter, coated inside with heparin. A drop of blood obtained from the tail of a nude mouse was allowed to enter the tube by capillarity, leaving at least 5mm unfilled at the top. The tubes were sealed with plasticine and centrifuged for 5 mins at 12,000 x g. Packed cell volume was determined by measuring the length of the tube packed with the red blood cells and the total length of the filled tube.

4.1.5 (c) Estimation of haemoglobin

The haemoglobin content of the blood of both tumour-bearing and non-tumour-bearing mice was determined by using Drabkin's cyanide-ferricyanide solution (Dacie and Lewis, 1975). The basis of the method involves dilution of blood in a solution containing potassium cyanide and potassium ferricyanide. Haemoglobin is converted under these conditions to cyanmethaemoglobin which can then be measured spectrophotometrically at 540nm. Blood (10µl of 1:2 dilution,diluted in PBS) was added to 100µl of Drabkins cyanide-ferricyanide solution (KCN 100mg/l; $KFeCN_3$ 300mg/l; pH 9.6) in a 96-well

micro-titre plate. The solution in each well was mixed and allowed to stand for 10 mins at room temperature to ensure the completion of the reaction. A pale yellow colour developed which was measured and compared with a haemoglobin standard curve (0-200μg). Readings were made against a blank (containing everything except the blood) in a spectrophotometer at a wavelength of 540nm.

4.1.6 Phenotypic characteristics of tumour cells

The phenotypic characteristics of differentiated and undifferentiated tumour cells were determined by their ability to produce ROI, measured by cytochrome C reduction and luminol-dependent CL, when these cells were stimulated with PMA (Sections 2.1.6 and 2.1.7). Lysosomal enzymes, such as acid phosphatase, specific and non-specific esterase were also measured, as described in Sections 2.1.10 and 2.1.11.

4.2 Results

4.2.1 The development of tumours in nude mice

The acronyms used to describe the different types of tumour cells are as follows:

HL60/T:- Tumour cells derived from nude mice transplanted with HL60 cells.

9DMSO/T:-Tumour cells derived from nude mice transplanted with 9 day DMSO-treated cells.

9BUT/T:- Tumour cells derived from nude mice transplanted with 9 day butyrate-treated cells.

No tumour development was observed in any of the control mice (groups two and three), irrespective of whether they were primed with cyclophosphamide. Pretreatment of nude mice with cyclophosphamide yielded tumour growth in 80-100% of animals transplanted with HL60 cells (3×10^6-4×10^7) (Table 4-4.3). In the absence of such priming (group 4) tumour development was observed in only 40% of the animals (i.e. 2 out of 5).

Table 4: Development of subcutaneous tumours by HL60 and differentiated cells in nude mice (3 x 10^6 cells were injected).

Cell type injected in mice	Number of tumour-bearing mice	Days taken for tumour development	Survival time of tumour-bearing mice (days)	Approximate tumour volume (cm^3)
HL60	4	18-20	55-65	1-4
9 day DMSO-treated cells	0	-	-	-
9 day butyrate-treated cells	1	18	33	6
1 day PMA-treated cells	3	18-20	30-35	3

Cells (3 x 10^6) were injected subcutaneously in five nude mice pretreated for 3 days with 250mg of cyclophosphamide. In control mice (5) serum-free medium (RPMI-1640, 0.2ml) was injected 3 days after priming with cyclophosphamide.

Table 4.1: Development of subcutaneous tumours by HL60 and differentiated cells in nude mice (4×10^7 cells were injected).

Cell type injected in mice	Number of tumour-bearing mice	Days taken for tumour development	Survival time of tumour-bearing mice (days)	Approximate tumour volume (cm^3)
HL60	5	10-12	20-30	12-15
9 day DMSO-treated cells	5	10-12	20-30	14-16
9 day butyrate-treated cells	5	10-12	20-30	14-16
1 day PMA-treated cells	ND	-	-	-

Cells (4×10^7) were injected subcutaneously in five nude mice pretreated for 3 days with 250mg of cyclophosphamide. In control mice (5) serum-free medium (RPMI-1640, 0.2ml) was injected 3 days after priming with cyclophosphamide. ND, not determined.

Table 4.2: Development of intraperitoneal tumours by HL60 and differentiated cells in nude mice (3 x 10^6 cells were injected).

Cell type injected in mice	Number of tumour-bearing mice	Days taken for tumour development	Survival time of tumour-bearing mice (days)	Number of solid tumours
HL60	5	25-35	45-60	5-7
9 day DMSO-treated cells	1	25	45	5-7

Cells (3 x 10^6) were injected intraperitoneally in five nude mice pretreated for 3 days with 250mg of cyclophosphamide. In control mice (5) serum-free medium (RPMI-1640, 0.2ml) was injected 3 days after priming with cyclophosphamide.

Table 4.3: Development of intraperitoneal tumours by HL60 and differentiated cells in nude mice (4 x 10^7 cells were injected).

Cell type injected in mice	Number of tumour-bearing mice	Days taken for tumour development	Survival time of tumour-bearing mice (days)	Number of solid tumours
HL60	5	10-12	15-30	6-7
9 day DMSO-treated cells	4	10-12	20-30	6-7
9 day butyrate-treated cells	4	10-12	20-30	6-7
1 day PMA-treated cells	4	15-18	20-30	6-7

Cells (4 x 10^7) were injected intraperitoneally in five nude mice pretreated for 3 days with 250mg of cyclophosphamide. In control mice (5) serum-free medium (RPMI-1640, 0.2ml) was injected 3 days after priming with cyclophosphamide.

No tumour development was observed when 9 day DMSO-treated cells (3×10^6) were injected subcutaneously to cyclophosphamide-primed mice, while 20-60% of the mice developed subcutaneous tumours when the same number of 9 day butyrate-treated and 1 day PMA-treated cells were given (Table 4). In all cases, subcutaneous, ovoid, pink tumours grew only at the site of injection and, if present, they were detected 18-20 days after transplantation as a palpable, moderately firm mass (Fig 4). Tumours and the overlying skin were mobile. Sarcomas developed a tan colour and in some cases a green hue could be observed over them. In the case of mice injected with butyrate and PMA treated-cells, severe cachexia was observed after 30 days of transplantation, and the tumours grew approximataly 1-6 cm^3 in volume (Table 4), after which the mice died. Mice transplanted with the same number of HL60 cells showed a similar pattern of tumour development, except that their survival time was significantly longer (55-65 days, Table 4). In all cases the tumours were not invasive. Cut sections revealed minimal vascularity and no metastases were observed at necropsy.

When the number of transplanted cells was increased by approximately ten-fold (4×10^7 cells), tumour development occurred in 100% of the mice injected with both the undifferentiated and differentiated (DMSO and butyrate-treated cells) HL60 cells (Table 4.1). The most striking difference from the lower cell dose was the similar rate of tumour development in all three types of tumours (Fig 4.2) and the reduced survival rate of the tumour-bearing mice (Table 4.1). The tumours that developed were comparatively larger than those induced by the smaller number of cells (Table 4 and 4.1). However, in both the cases, subcutaneous tumours which developed on transplantation of the mice with chemically-differentiated cells grew faster than those tumours seen in mice injected with undifferentiated HL60 cells (Figs 4.1 and 4.2).

Intraperitoneal transplantation of undifferentiated HL60 cells (3×10^6) produced tumour development in 100% of the animals (Table 4.2). In all cases the pattern of intraperitoneal tumour growth was very different to that found with subcutaneous injection. Within 21 days of cell transplantation, the mice exhibited a swollen peritoneal cavity which, if excised during that period, displayed no solid tumours but only ascitic

Fig 4: Development of a subcutaneous tumour by HL60 cells in nude mice at the site of injection.

Fig 4

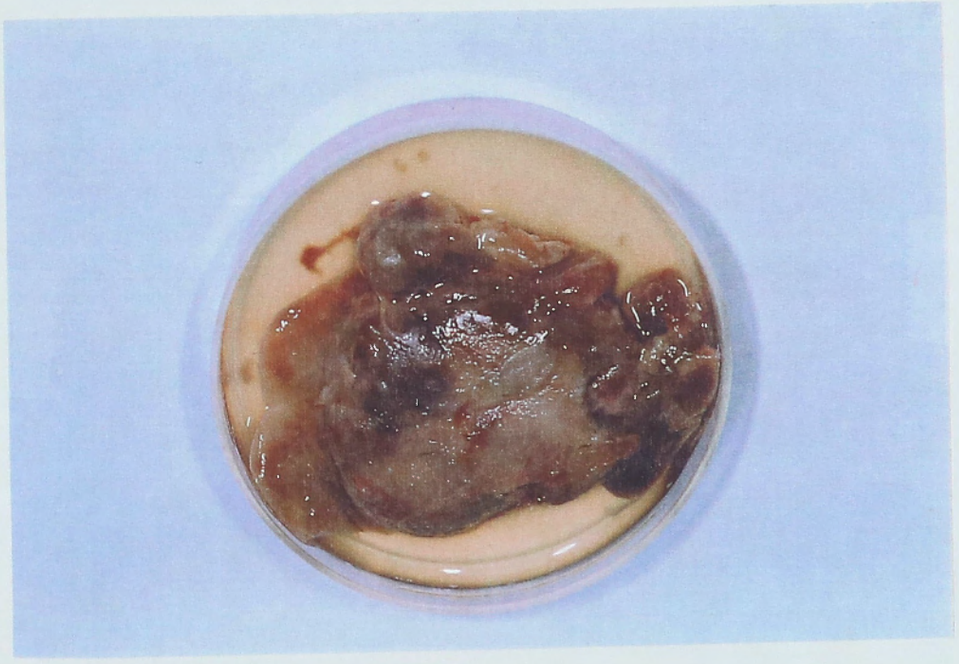


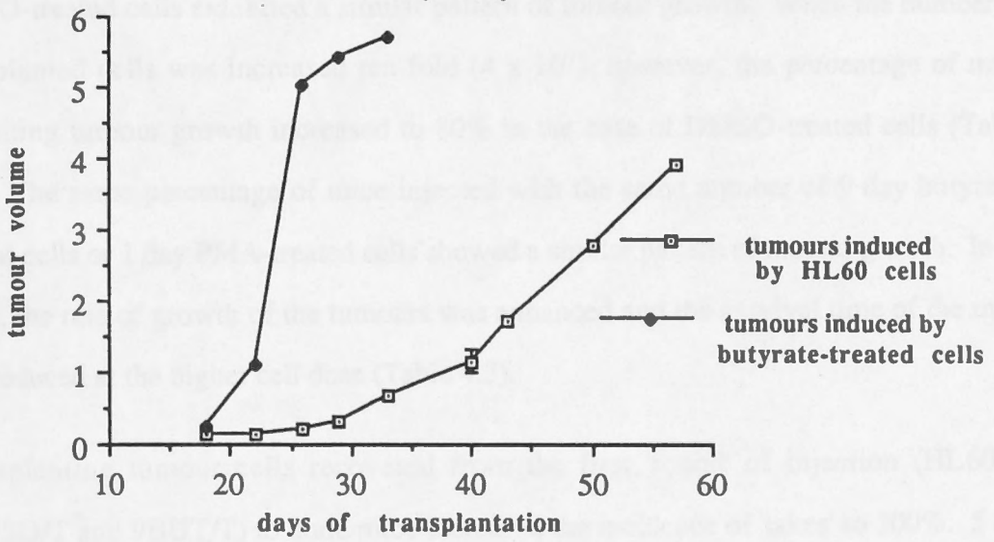
Fig 4.1: Growth rate of tumours developed in nude mice by HL60 and 9 day butyrate-treated cells.

Tumour volumes were measured as described in the Materials and Methods section and are expressed as cm^3. 3×10^6 cells were injected subcutaneously in each case. For the tumours induced by HL60 cells , measurements were made on three different mice and the results are means $\pm$ S.E.M. of the measurements performed. In the case of 9 day butyrate induced HL60 cells, tumour measurement was performed on only one mouse exhibiting tumour growth.

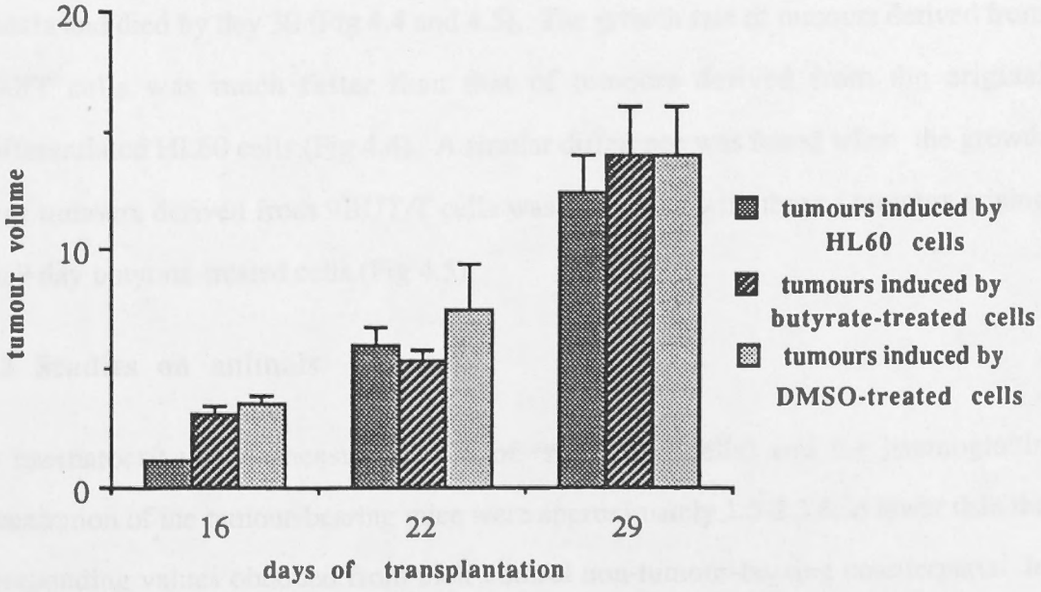
Fig 4.2: Growth rates of tumours developed in nude mice by HL60, 9 day butyrate-treated and 9 day DMSO-treated cells.

4×10^7 cells were injected subcutaneously in each case. Tumour volumes were measured as described in the Materials and Methods section and are expressed as cm^3. Tumour measurements were made on three different mice and the results are means $\pm$ S.E.M. of the measurements performed.

(4.1) Growth rate of tumours developed in nude mice by HL60 and 9 day butyrate-treated cells



(4.2) Growth rate of tumours developed in nude mice by HL60, 9 day butyrate and DMSO-treated cells



fluid. If the mice were allowed to live for another 7-10 days and then killed, however, besides ascitic carcinosis, 5-7 small solid tumours were observed in the peritoneal cavity just above the bladder (Fig 4.3). Autopsy of the dead mice revealed no metastases in other parts of the body. Only 20% of the mice injected intraperitoneally with 9 day DMSO-treated cells exhibited a similar pattern of tumour growth. When the number of transplanted cells was increased ten-fold (4×10^7), however, the percentage of mice exhibiting tumour growth increased to 80% in the case of DMSO-treated cells (Table 4.3). The same percentage of mice injected with the same number of 9 day butyrate-treated cells or 1 day PMA-treated cells showed a similar pattern of tumour growth. In all cases, the rate of growth of the tumours was enhanced and the survival time of the mice was reduced at the higher cell dose (Table 4.3).

Transplanting tumour cells recovered from the first 'round' of injection (HL60/T, 9DMSO/T and 9BUT/T) to nude mice increased the incidence of 'takes' to 100%. 5 out of 5 mice given as few as 3×10^6 cells developed solid sarcomas like their parental cells at the site of injection. By day 10 visible tumour growth was observed and by day 25 the volume of the tumours had reached approximately 4 cm^3. Mice developed severe cachexia and died by day 30 (Fig 4.4 and 4.5). The growth rate of tumours derived from HL60/T cells was much faster than that of tumours derived from the original undifferentiated HL60 cells (Fig 4.4). A similar difference was found when the growth rate of tumours derived from 9BUT/T cells was compared with that of tumours arising from 9 day butyrate-treated cells (Fig 4.5).

4.2.2 Studies on animals

The haematocrit value (measured as % of red blood cells) and the haemoglobin concentration of the tumour-bearing mice were approximately 1.5-2.3 fold lower than the corresponding values obtained from their control non-tumour-bearing counterparts. In those mice bearing tumours of undifferentiated HL60 cell origin, these values were significantly lower (Table 4.4) than those obtained when the tumours were derived from butyrate and DMSO-treated cells. The leukocyte counts in both the tumour-bearing and

Fig 4.3: Development of intraperitoneal tumours in the peritoneal cavities of nude mice injected with HL60 cells.

Fig 4.3

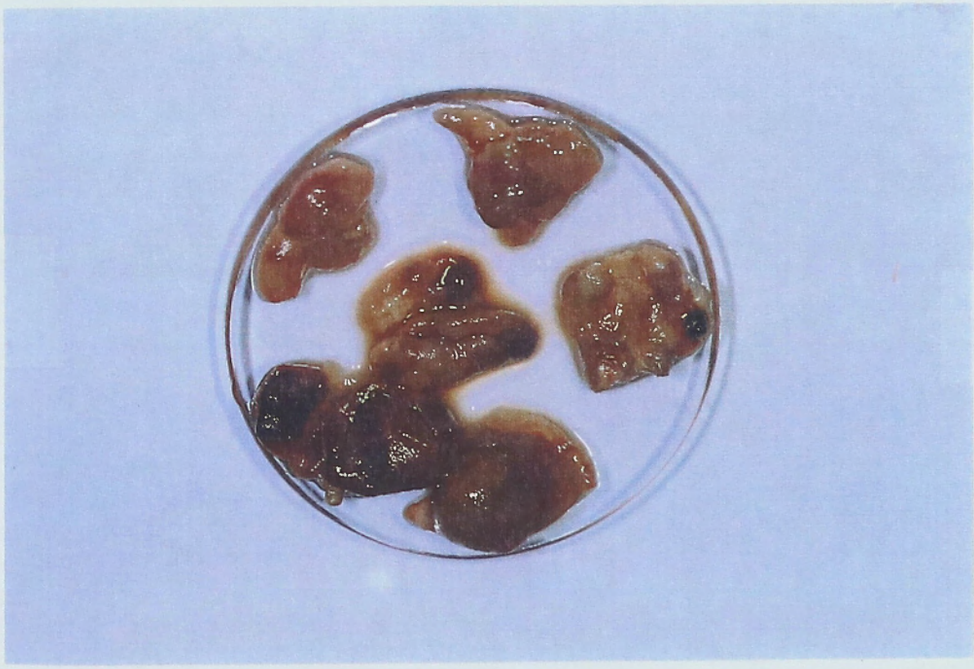


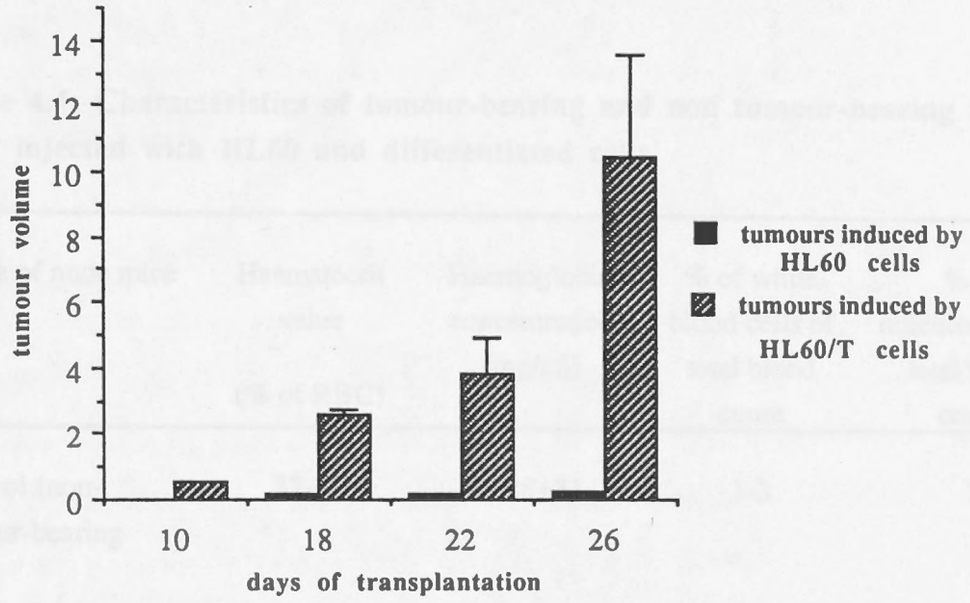
Fig 4.4: Growth rates of tumours developed in nude mice by HL60 and HL60/T cells.

Tumour volumes were measured as described in the Materials and Methods section and are expressed as cm^3. In each case tumour measurements were made on three different mice and the results are means $\pm$ S.E.M. of the measurements performed .

Fig 4.5: Growth rates of tumours developed in nude mice by 9 day butyrate-treated and 9BUT/T cells.

Tumour volumes were measured as described in the Materials and Methods section and are expressed as cm^3. For the 9BUT/T induced-cells, tumour measurements were performed on three different mice and the results are means $\pm$ S.E.M. of the measurements performed. In the case of 9 day butyrate-induced cells tumour measurements were performed on only one mouse exhibiting tumour growth.

(4.4) Growth rate of tumours developed in nude mice by HL60 and HL60/T cells



(4.5) Growth rate of tumours developed in nude mice by 9 day butyrate-treated and 9BUT/T cells

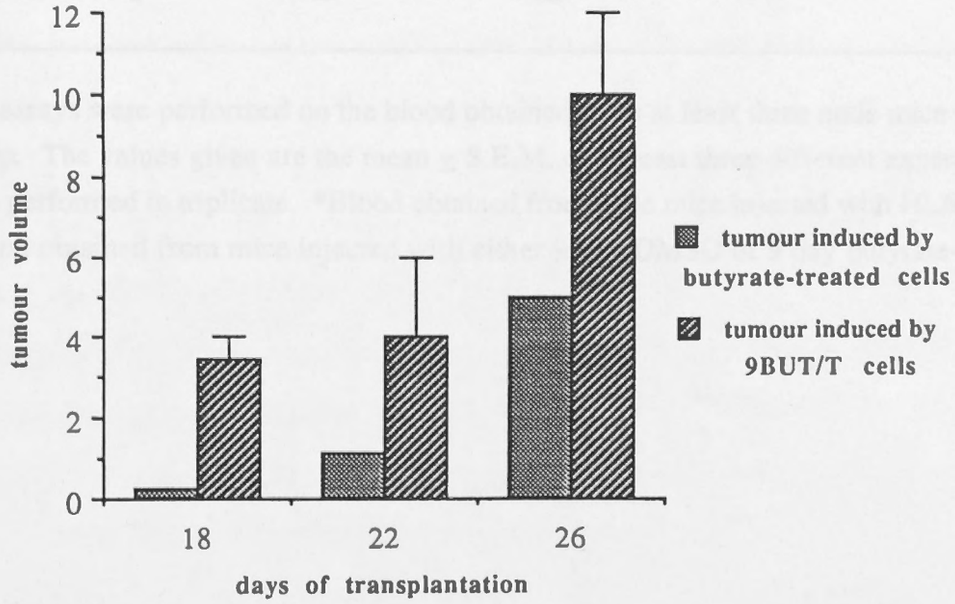


Table 4.4: Characteristics of tumour-bearing and non tumour-bearing nude mice injected with HL60 and differentiated cells.

Type of nude mice	Haematocrit value (% of RBC)	Haemoglobin concentration (mg/ml)	% of white blood cells of total blood count	% of reticulocytes of total blood count
Control (non-tumour-bearing mice)	33±1	228±31	1-3	1-3
Tumour-bearing mice*	21±1	98±12	1-2	6-12
Tumour-bearing# mice	23±3	138±17	1-2	1-2

The assays were performed on the blood obtained from at least three nude mice in each group. The values given are the mean ± S.E.M. of at least three different experiments, each performed in triplicate. *Blood obtained from nude mice injected with HL60 cells; #blood obtained from mice injected with either 9 day DMSO or 9 day butyrate-treated cells.

non-bearing animals remained the same. However, the percentage of reticulocytes in HL60-injected tumour-bearing mice was twice that of the non-tumour-bearing mice and those tumour-bearing mice injected with chemically-treated cells.

4.2.3 Morphological and phenotypic characteristics of tumour cells

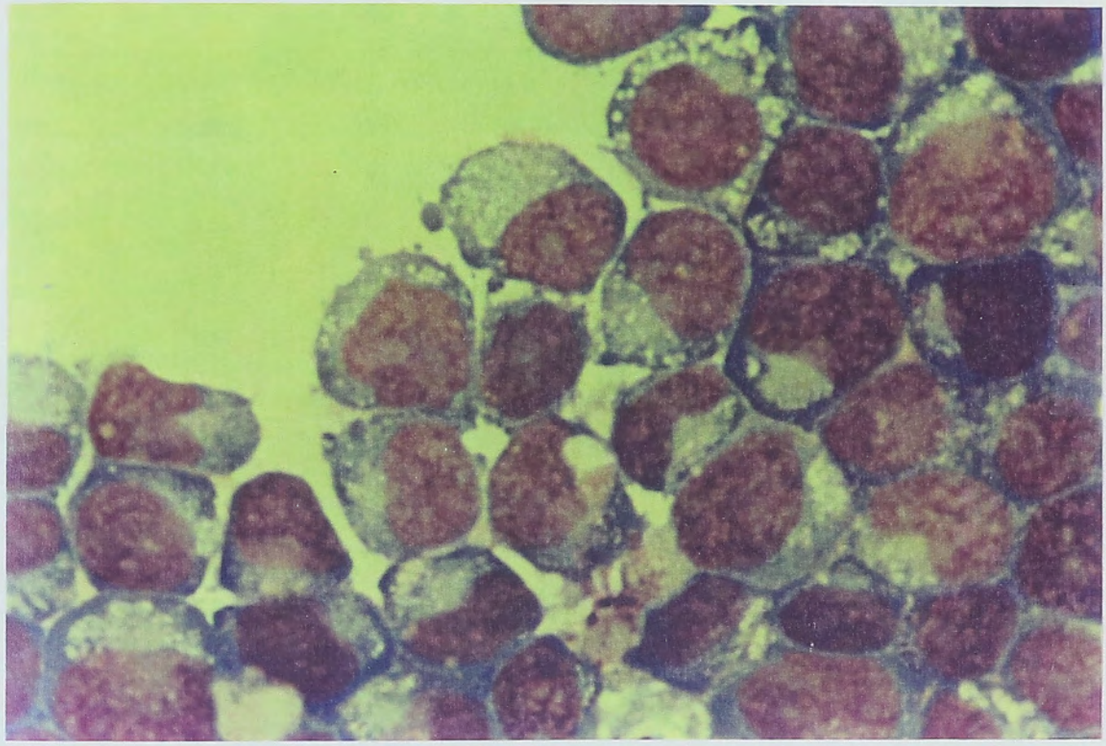
Morphological examination of tumour cells recovered from the nude mice (HL60/T, 9DMSO/T and 9BUT/T) showed strong resemblance to their parental HL60 cells. Except for the number of vacuoles, which were present in increased numbers (compared to cultured HL60 cells) in the cytoplasm and the nucleus of both 9DMSO/T and 9BUT/T tumour cells, the rest of the cellular morphology remained relatively unaltered after tumourigenesis (Fig 4.6). The phenotypic characteristics of tumour cells were assessed by measuring their capacity to produce ROI on stimulation with PMA. Other measurements were made of the level of secreted lysosomal enzymes (acid phosphatase, specific and non-specific esterase) and the extent of differentiation of tumour cells in response to DMSO and butyrate.

ROI production was measured from the rate of the luminol-dependent CL response and the rate of reduction of exogenous cytochrome C. Both methods showed that tumour HL60/T cells had higher responses than the cultured undifferentiated HL60 cells (Fig 4.7 and 4.8). On the other hand, 9BUT/T and 9DMSO/T cells exhibited approximately the same level of response as the cultured HL60 cells (Fig 4.7 and 4.8). Differentiating the tumour cells (HL60/T, 9BUT/T and 9DMSO/T) for a further 9 days with DMSO and butyrate after their removal from the mice enhanced luminol-dependent CL responses and cytochrome C reduction in all tumour cells, but the response was still lower than that of the original 9 day DMSO and butyrate-differentiated HL60 cells (Fig 4.9-4.12). Nevertheless, differentiated HL60/T cells still exhibited responses that were comparatively higher than those of differentiated 9BUT/T and 9DMSO/T cells. These results are consistent with the lower levels of specific and non-specific esterase found in the DMSO and butyrate-treated tumour cells (Table 4.5) compared to their differentiated HL60 counterparts and tumour HL60/T cells.

Fig 4.6: Light microscopy of tumour cells.

(a) HL60/T cells; and **(b)** 9DMSO/T cells. The cells were examined using oil under x 1000 maginification.

Fig 4.6 (a)



1000

Fig 4.6 (b)

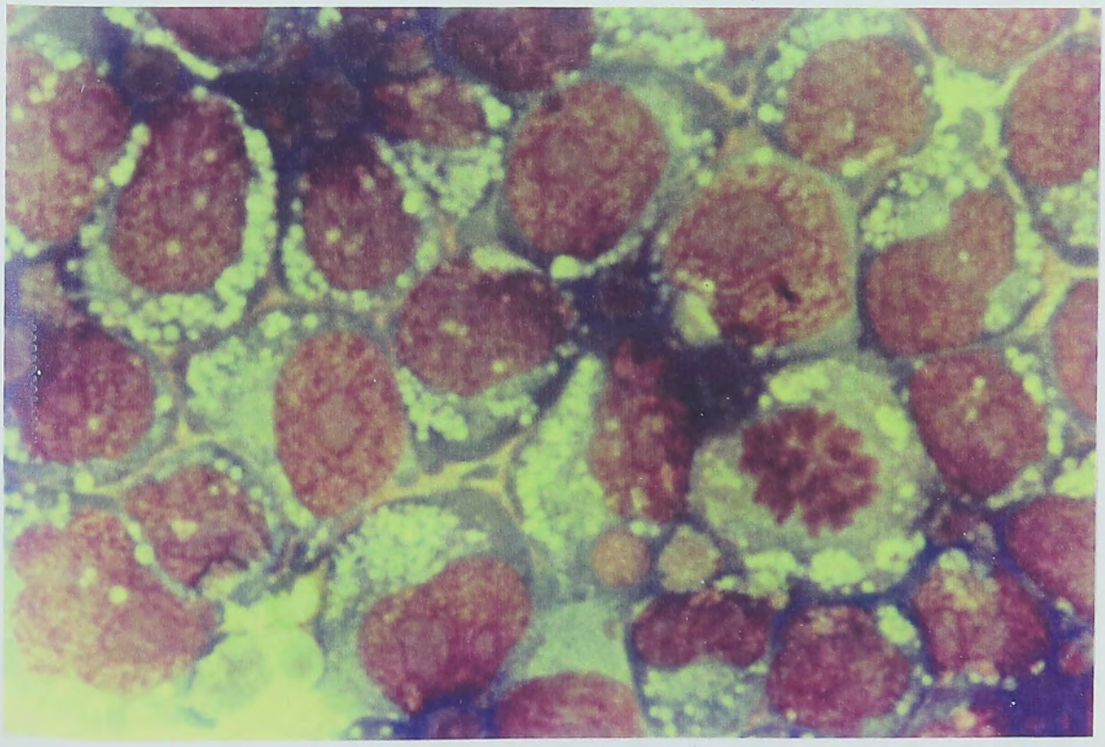


Fig 4.6: Light microscopy of tumour cells.

(c) 9BUT/T cells. The cells were examined using oil under x 1000 maginification.

Fig 4.6 (c)

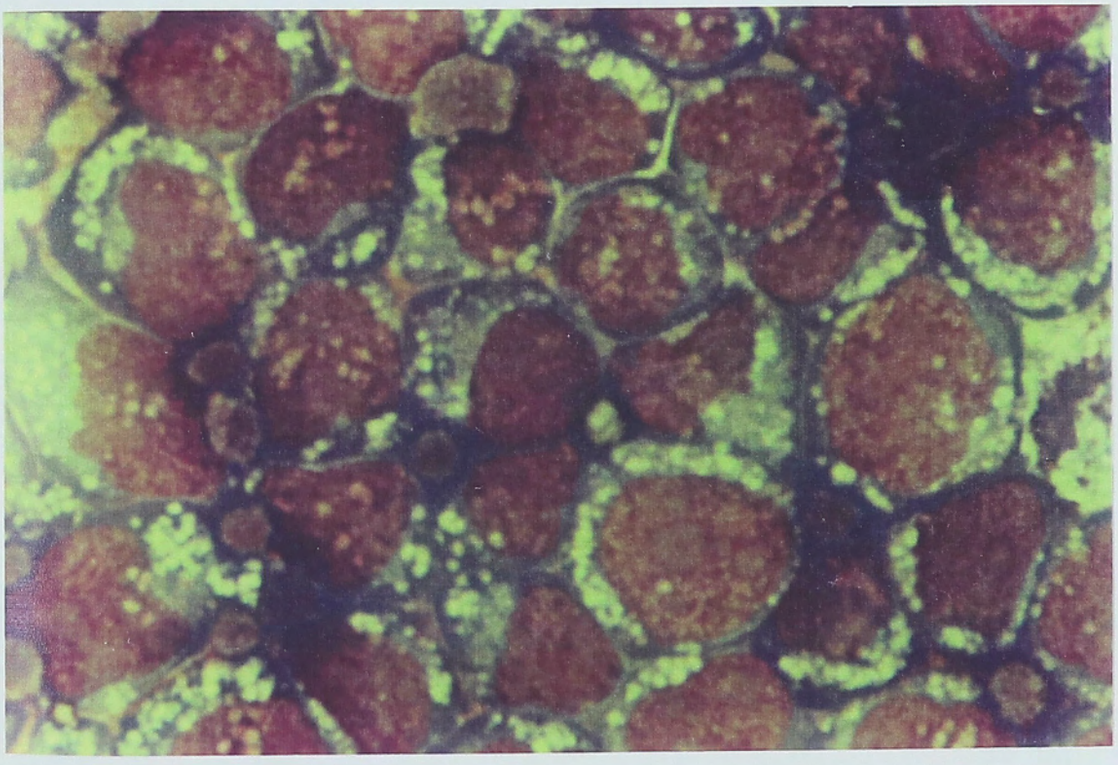


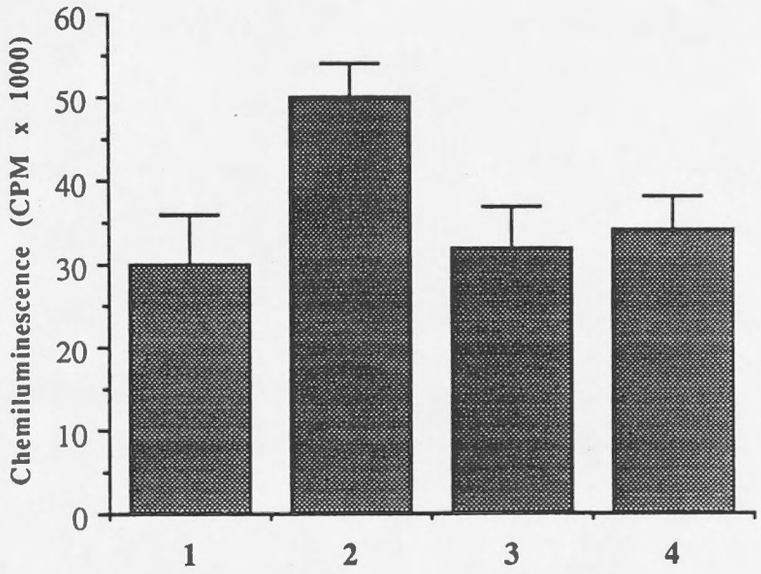
Fig 4.7: Peak CL response given by HL60 and tumour cells.

(1) response given by cultured HL60 cells; (2) response given by HL60/T cells; (3) response given by 9DMSO/T cells; and (4) response given by 9BUT/T cells. In each case 1 x 10^6 cells were taken and the response was measured in the presence of luminol (220μM) and HRP(9U/ml) after stimulating the cells with PMA (1μM). The remaining conditions of the experiment are described in the Materials and Methods section. Each value is the mean ± S.E.M. of at least three separate experiments, each performed in duplicate.

Fig 4.8: Superoxide production by HL60 and tumour cells.

(1) response given by cultured HL60 cells; (2) response given by HL60/T cells; (3) response given by 9DMSO/T cells; and (4) response given by 9BUT/T cells. In each case 2 x 10^6 cells were taken and the superoxide production was measured by the reduction of cytochrome C after stimulating the cells with PMA (1.5μg). The remaining conditions of the experiment are described in the Materials and Methods section. Each value is the mean ± S.E.M. of at least three separate experiments, each performed in duplicate.

(4.7) Peak CL response by HL60 and tumour cells



(4.8) Superoxide produced by HL60 and tumour cells

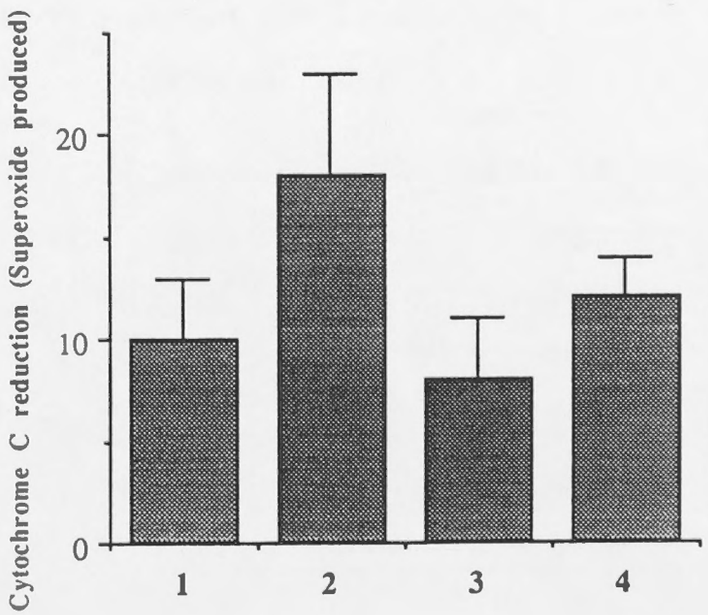


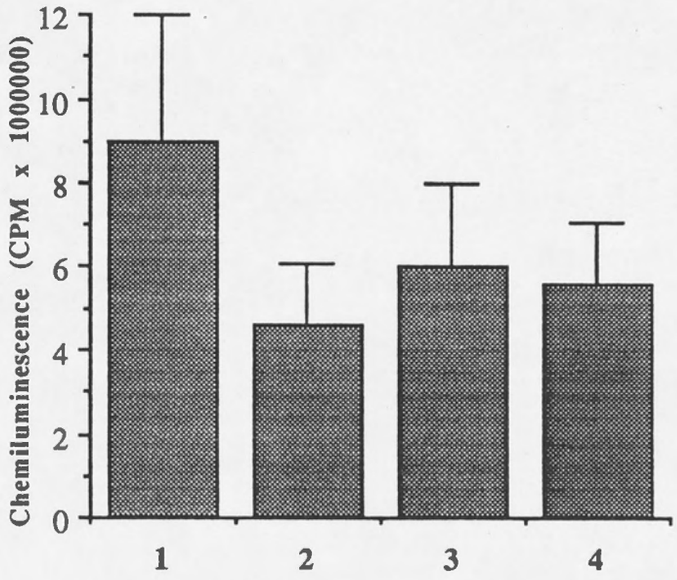
Fig 4.9: Peak CL response after 9 day DMSO treatment of HL60 and tumour cells.

(1) response given by 9 day DMSO-treated HL60 cells; (2) response given by 9 day DMSO-treated HL60/T cells; (3) response given by 9 day DMSO-treated 9DMSO/T cells; and (4) response given by 9 day DMSO-treated 9BUT/T cells. In each case 1 x 10^6 cells were taken and the response was measured in the presence of luminol (220μM) and HRP (9U/ml) after stimulating the cells with PMA (1μM). The remaining conditions of the experiment are described in the Materials and Methods section. Each value is the mean ± S.E.M. of at least three separate experiments, each performed in duplicate.

Fig 4.10: Superoxide produced after 9 day DMSO treatment of HL60 and tumour cells.

(1) response given by 9 day DMSO-treated HL60 cells; (2) response given 9 day DMSO-treated HL60/T cells; (3) response given by 9 day DMSO-treated 9DMSO/T cells; and (4) response given by 9 day DMSO-treated 9BUT/T cells. In each case 2 x 10^6 cells were taken and the superoxide production was measured after stimulating the cells with PMA (1.5μg). The remaining conditions of the experiment are described in the Materials and Methods section. Each value is the mean ± S.E.M. of at least three separate experiments, each performed in duplicate.

(4.9) Peak CL response after 9 day DMSO treatment of HL60 and tumour cells



(4.10) Superoxide produced after 9 day DMSO treatment of HL60 and tumour cells

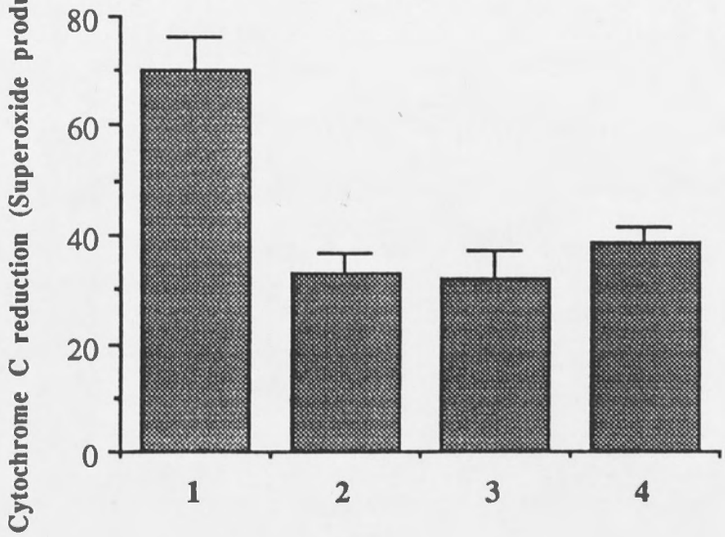


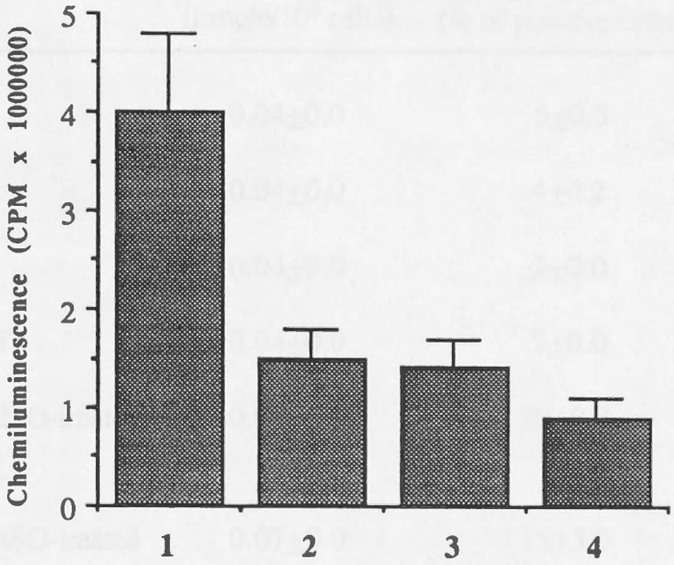
Fig 4.11: Peak CL response after 9 day butyrate treatment of HL60 and tumour cells.

(1) response given by 9 day butyrate-treated HL60 cells; (2) response given by 9 day butyrate-treated HL60/T cells; (3) response given by 9 day butyrate-treated 9DMSO/T cells; and (4) response given by 9 day butyrate-treated 9BUT/T cells. In each case 1 x 10^6 cells were taken and the response was measured in the presence of luminol (220μM) and HRP (9U/ml) after stimulating the cells with PMA (1μM). The remaining conditions of the experiment are described in the Materials and Methods section. Each value is the mean ± S.E.M. of at least three separate experiments, each performed in duplicate.

Fig 4.12: Superoxide produced after 9 day butyrate treatment of HL60 and tumour cells.

(1) response given by 9 day butyrate-treated HL60 cells; (2) response given by 9 day butyrate-treated HL60/T cells; (3) response given by 9 day butyrate-treated 9DMSO/T cells; and (4) response given by 9 day butyrate-treated 9BUT/T cells. In each case 2 x 10^6 cells were taken and the superoxide production was measured after stimulating the cells with PMA (1.5μg). The remaining conditions of the experiment are described in the Materials and Methods section. Each value is the mean ± S.E.M. of at least three separate experiments, each performed in duplicate.

(4.11) Peak CL response after 9 day butyrate treatment of HL60 and tumour cells



(4.12) Superoxide produced after 9 day butyrate treatment of HL60 and tumour cells

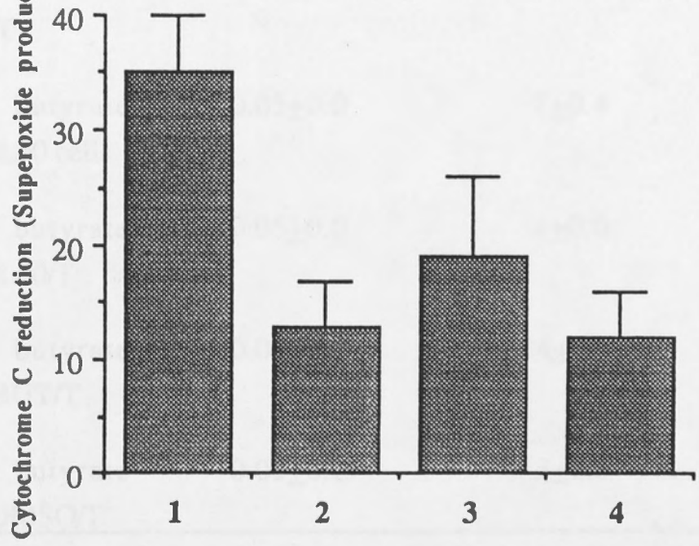


Table 4.5 Lysosomal enzyme levels in HL60 and tumour cells.

Cell Type	Acid phosphatase (μmole/10^6 cells)	Specific esterase (% of positive cells)	Non-specific esterase (% of positive cells)
HL60	0.04±0.0	5±0.5	5±0.5
HL60/T	0.04±0.0	4±0.2	7±0.8
9BUT/T	0.04±0.0	2±0.0	7±0.1
9DMSO/T	0.04±0.0	2±0.0	8±1.0
9 day DMSO-treated HL60	0.07±0.0	20±0.9	9±1.0
9 day DMSO-treated HL60/T	0.07±0.0	15±5.0	12±4.0
9 day DMSO-treated 9BUT/T	0.04±0.0	6±0.5	15±1.0
9 day DMSO-treated 9DMSO/T	0.04±0.0	8±0.9	17±2.0
9 day butyrate-treated HL60 cells	0.05±0.0	7±0.4	40±2.0
9 day butyrate-treated HL60/T	0.05±0.0	4±0.0	30±8.0
9 day butyrate-treated 9BUT/T	0.04±0.0	4±0.5	30±6.0
9 day butyrate-treated 9DMSO/T	0.05±0.0	3±0.0	30±8.0

The assays were performed on three different tumours (in each case) in duplicate. The values given are means ± S.E.M. of at least three different experiments, each performed in duplicate.

4.3 Discussion

The resistance of the host, *in vivo*, to progressive tumour growth has been attributed mainly to the presence of mature T cells with specific receptors for tumour-associated antigens. However, even though the central role of mature T cells has been stressed, there are at least three other types of effector cells (or cellular mechanisms) which contribute to cell-mediated cytotoxicity against tumour cells (Cerottini and Brunner, 1974; Shellam and Hogg, 1977). These are: (1) antibody-dependent cytotoxic cells, which bear surface receptors for the F_c portions of IgG molecules that interact with antigenic structures on the surfaces of target cells; (2) activated macrophages; and (3) 'natural' cell-mediated cytotoxicity associated with natural killer cell (NK) activity. Nude mice more than three weeks old show considerable NK cell activity which reaches its peak level between five to eight weeks. Thereafter, there is a gradual decline in activity to lower levels, which persists indefinitely (Herberman *et al.*, 1975; Kiessling *et al.*, 1975). NK cell activity against a particular target cell is considerably higher in nude mice than in conventional thymus-bearing mice and it is thought to be related inversely to the degree of thymic function (Herberman and Holden, 1978). Treatment of nude mice with cyclophosphamide results in a considerable decrease in NK cell activity, with a reduction of greater than 70% at one to three days after administration of 110mg/Kg of body weight *in vivo* (Dr. J.Y.Djeu, unpublished result, data cited in Herberman and Holden, 1978). Treatment of nude mice with cyclophosphamide not only results in the inhibition of NK cell activity but also causes a considerable decrease in antibody-dependent cell-mediated cytotoxicity (Herberman and Holden, 1978). The method of tumour induction used in the present experiments was based on these observations and gave results consistent with the data presented by Dr. J.Y.Djeu : a single priming dose of cyclophosphamide (250mg) enhanced the efficiency of subcutaneous induction of human tumours in nude mice by doubling the yield from 40% without cyclophosphamide priming to 80% after priming. This suggests that nude mice vary in their ability to mount antitumour activity against xenogeneic tumour cells (Fogh *et al.*, 1977) and supports the idea that both NK cells and antibody-dependent cell-mediated cytotoxicity [both of which are inhibited by

cyclophosphamide (Herberman and Holden, 1978)] are involved in the rejection of xenogeneic tumours in nude mice.

The incidence of tumours induced in nude mice by undifferentiated HL60 cells and their chemically-differentiated counterparts (i.e. DMSO, butyrate and PMA-treated cells) depended on the number of cells injected and the site of injection (Tables 4-4.3). At low numbers of transplanted cells (3×10^6), the incidence of tumour growth was quite low, especially when the cells had been chemically-differentiated prior to injection. DMSO-treated cells (3×10^6), for example, failed to show any tumour growth at all when injected subcutaneously into nude mice, while 20-60% of the mice exhibited subcutaneous tumours when butyrate and PMA-treated cells were transplanted (Table 4). If the number of cells injected was increased by approximately ten-fold, however, the incidence of tumour growth increased to 100%, irrespective of the prior treatment of the transplanted cells. Tumours derived from the chemically-differentiated cells may originate from the small proportion of inducer-resistant cells (i.e. cells resistant to differentiation by DMSO, butyrate and PMA) which persist in the vast majority of differentiated cells. Earlier workers have shown that 5-10% of the total population of undifferentiated HL60 cells consists of cells which are refractory to differentiation by inducers (Bamberger *et al.*, 1978; Leftwich, 1987). Increasing the absolute number of transplanted cells also increases the number of inducer-resistant cells present in the injected population to a point which may exceed the threshold necessary to establish tumour growth. The rate of growth of such tumours was faster than that of HL60-induced tumours (Fig 4.1 and 4.2) and the survival time of the tumour-bearing mice was shorter than that of mice bearing conventional HL60-induced tumours. At this particular threshold, the enrichment of resistant cells with high intrinsic tumourigenicity in a background population of non-tumourigenic differentiated cells, may be responsible for the more rampant tumour growth.

In all cases of subcutaneous transplantation a solid tumour mass was observed only at the site of injection (Fig 4). No distant metastases were observed in any other organ on autopsy of the dead mice. In some cases, the tumours displayed green coloration which

may be related to the production of myeloperoxidase by host neutrophils accumulating at the site of the injected cells. A similar observation was made by Potter *et al.* (1985), who referred to the induced HL60 tumours in nude mice as 'granulocytic sarcomas' because of their resemblance to human tumours of the same origin.

The nature of tumour growth observed in nude mice on intraperitoneal transplantation of HL60 and chemically-differentiated cells was very different to that seen with subcutaneous injection. The different site of injection clearly influenced the subsequent pattern of tumour growth. The occurrence of both ascitic and solid tumours following intraperitoneal injection could be the result of transient functional changes in tumour cells associated with their establishment in a different environment (Weiss, 1980). Several investigators, using different tumour cell lines, have demonstrated that intraperitoneal injection in nude mice can be followed immediately or gradually by ascitic carcinoma which, in some cases, may lead to the growth of solid tumour masses in the peritoneal cavity with or without any incidence of further metastases (Hirohashi *et al.*, 1976; Takahashi *et al.*, 1978; Kyriazis *et al.*, 1978; Sordat and Bogenmann, 1980). Intraperitoneal injection of leukaemic and chemically-differentiated HL60 cells was followed by the development of ascitic tumours. Within 10-20 days of intraperitoneal transplantation, mice displayed a greatly distended peritoneal cavity. Autopsy of these mice revealed a large number of freely floating and rapidly growing cells in the peritoneal cavity. In the surviving mice the ascitic carcinoma was replaced gradually by small solid tumour masses (Fig 4.3) so that, by 30-45 days after tumour cell injection, six to seven solid tumours were observed in the peritoneal cavity just above the bladder. The tumours resembled a chain of beads. On autopsy no metastases were observed in any other organ of the tumour-bearing mice. The lack of metastatic spread is an intriguing characteristic of human tumours transplanted in immunodeficient nude mice, and the tumours formed by HL60 cells appear to behave like other solid tumours. The absence of metastases may be related to the presence of 'activated' macrophages with tumouricidal activity (Meltzer, 1976) or it may occur in response to the rebound in NK cell activity which happens approximately ten days after the initial cyclophosphamide priming (J.Y. Djeu unpublished

result, data cited in Herberman and Holden, 1978). Treatment of nude mice with goat anti-mouse Ig raised specifically against myelogenous leukaemic cells of the K562 cell line prevented the growth of, or destroyed, well-developed myelosarcomas made up of K562 cells growing in nude mice (Latif *et al.*, 1980). Similarly, monoclonal antibodies of IgG2a isotype specifically inhibited the growth of human tumours in nude mice (Herlyn and Koprowski, 1982). The mechanism by which antibody inhibits the proliferation of tumour cells *in vivo* probably involves antibody-dependent macrophage-mediated cytotoxicity (Herlyn and Koprowski, 1982). Retransplanting the tumour cells in nude mice (using both subcutaneous and intraperitoneal inoculation), produced tumours in 100% of the mice irrespective of the source from which the cells were derived. Even though the pattern of tumour growth induced by transplanted tumour cells and cultured cells was the same, the growth rate of the former tumour cells was much faster than that of tumours induced by cultured cells (Figs 4.4 and 4.5).

Studies on the blood profile of the mice showed that the haemotocrit value in all tumour-bearing mice was significantly lower than that of the non-tumour-bearing controls (Table 4.4). This is consistent with the lower haemoglobin concentration in the tumour-induced mice compared with their control counterparts. The lower haematocrit value and haemoglobin concentration in the tumour induced-mice may be related to the cachexia (observed by the wasting of muscles in the tumour-bearing mice) which is a general characteristic of mice in this condition. Although all tumour-bearing mice exhibited the same percentage of white blood cell counts as their control partners, the percentage of reticulocytes in some tumour-bearing mice (notably those derived from undifferentiated HL60 cells) was approximately two-fold higher than those of the control mice. The lack of enhancement of the white blood cell count in the tumour-bearing mice is indicative of the absence of leukaemia in these mice. The increased percentage of reticulocytes in some of the tumour-bearing mice is possibly associated with anaemia-related cachexia. However, the lack of anaemia in other tumour-bearing mice (e.g. those derived from the chemically-differentiated cells) is puzzling and cannot be explained.

Morphological examination of tumour cells recovered from the nude mice (HL60/T, 9DMSO/T and 9BUT/T) showed a strong resemblance to cultured HL60 cells (Fig 4.6), yet studies of their phenotypic characteristics gave results quite different to the parental HL60 cells, indicating that they have acquired new properties in response to the environment *in vivo* . Phenotypic characteristics of the tumour cells were assessed from their respiratory burst activity (measured as ROI production), their lysosomal enzyme content and the retention or loss of their differentiating capabilities. HL60/T cells showed a higher degree of respiratory burst activity (measured as peak luminol-dependent CL and superoxide anion production from superoxide dismutase-inhibitable cytochrome C reduction) than 9DMSO/T, 9BUT/T and cultured HL60 cells, suggesting that they have retained some differentiated properties compared with the other tumour cells and cultured HL60 cells tested (Figs 4.7 and 4.8). The respiratory burst activities of 9DMSO/T and 9BUT/T cells were the same as those of cultured HL60 cells, which is consistent with the similarity of their morphological properties and lysosomal enzyme levels. The differentiated properties of HL60 and other leukaemic cells proliferating in nude mice have been reported by Machado *et al.* (1984). Similarly, Giovanella and Stehlin (1974) reported that cell populations derived from tumours developed in nude mice were more differentiated than their parental cultured cells. In a large series of tumours transplanted directly from surgical specimens, Fogh and Hajdu (1975) observed that 25% of the tumours developed in nude mice had cells which showed a higher degree of differentiation than the parent tumours. It appears, therefore, that the host environment is not only favourable for preserving the basic features of the tumour cells but it can also induce alterations consistent with some degree of adaptive maturation. Mouse tissues are known to be capable of synthesizing natural biological factors (such as CSFs) that can affect the differentiation of cultured cells proliferating *in vivo*. Since these factors are T-lymphocyte independent, the athymic condition of the host should not interfere with their production. The lack of differentiation observed in 9DMSO/T and 9BUT/T cells may be due to their greater requirement than HL60/T cells for the differentiating factors, which may exceed the concentration the mouse tissue is capable of producing.

Differentiation of the tumour cells with DMSO and butyrate increased the degree of maturation of these cells [as evidenced by their enhanced CL response, cytochrome C reduction and lysosomal enzyme levels (Figs 4.9-4.12 and Table 4.5)], but the maturation did not proceed to the same extent as in cultured HL60 cells. These observations are consistent with the emergence of a phenotypic dissimilarity between the tumour cells and their cultured cell progenitors, even though they may look the same morphologically. Phenotypic variation in tumour cells may arise as a result of chromosomal alterations induced by the changed environment of the host *in vivo*. Studies on HL60/MRI, a cell line established from a transplantable HL60 tumour in nude mice, showed chromosomal alterations compared to the progenitor HL60 cells (Imaizumi *et al.*, 1987), in consequence of which the proto-oncogene *c-myc* of HL60/MRI was amplified about two-fold less in genomic DNA and expressed about two-fold less at the transcriptional level than in the original HL60 cells (Imaizumi and Breitman, 1988). Moreover, cells of the HL60/MRI line were more sensitive to RA differentiation than cultured HL60 cells and they differentiated to monocytoid-like cells compared with the granulocytic differentiation induced in HL60 cells (Imaizumi *et al.*, 1987). The altered behaviour of tumour cells (HL60/MRI) may be indicative in this case of a mutation resulting in a change of gene expression.

The results presented in this Chapter demonstrate that differentiation of HL60 cells by DMSO, butyrate and PMA is incomplete, as the differentiated cells remained capable of developing into tumours in nude mice. Tumourigenicity induced by differentiated cells, especially when they were injected at low concentrations (3×10^6), was much less severe (as seen from the percentage of tumour-bearing mice) than that induced by the leukaemic HL60 cells. Both the leukaemic and differentiated cells failed to cause leukaemogenicity in nude mice. Even though the tumour cells looked similar morphologically to the cultured HL60 cells, these cells showed some phenotypic variation which might represent an effect of the environment of the host *in vivo* on the tumour cells.

Chapter 5: Glycolytic, glutaminolytic and pentose-phosphate pathways in HL60 and DMSO-treated cells

5 Introduction

The induction of differentiation of the human promyelocytic leukaemic cell line HL60 by DMSO is accompanied by the appearance of several functional characteristics of mature granulocytes (Collins *et al.*, 1978). These include, among others, the expression of surface receptors for the synthetic oligopeptide chemotactic factor (Fontana *et al.*, 1980) and acquisition of the capacity to express chemotactic (Collins *et al.*, 1979), phagocytic (Newburger *et al.*, 1979) and oxidative responses (Ahmed *et al.*, 1991) when challenged with appropriate stimuli. Although much is known about the morphological and functional changes that accompany the induction of differentiation of HL60 and other leukaemic cells (Harris & Ralph, 1985), very little work has been done on the changing pattern of substrate utilization that occurs in parallel. Hence the metabolism of two major fuels glucose and glutamine was investigated in this study.

Aim of the study

The main aim of the project is to describe the metabolic nature of these malignant cells and their differentiated counterparts in order to investigate the way by which these cells might over-produce intermediates and products like other malignant cells (Eigenbrodt *et al.*, 1985). It is proposed that enhanced levels of some of these intermediates ultimately promote and account for increased nucleic acid and protein production that leads to malignancy. Hence, it was considered important to investigate systematically the metabolic characteristics of the two major fuels, glucose and glutamine, that are used by these cells. To this end, the maximum catalytic activities of some of the key enzymes involved in glucose and glutamine metabolism have been measured, together with the rates of utilization of the two substrates by undifferentiated and DMSO-differentiated HL60 cells (Sections 5.1.4, 5.1.5, 5.1.7 and 5.1.8). The metabolic fates of glucose and glutamine have been investigated by measuring the intermediates and products that accumulate when cells are incubated with these substrates, separately and together. Such data will help in establishing the optimum levels of glucose and glutamine for the required growth and differentiation. This study was also extended to measure the rates of

utilization, oxidation and conversion of [1-^{14}C]-glucose and [6-^{14}C]-glucose to $^{14}CO_2$. This will help in the measurement of the pentose phosphate pathway contribution to glucose metabolism (Section 5.1.4). The nature of this pathway was also examined (Sections 5.1.2 and 5.1.6). Pentose phosphate pathway plays an important role in leukaemic cell differentiatiation since it is the only known pathway whereby cells produce ribose 5-phosphate necessary for PRPP, and hence, purine/pyrimidine nucleotides and nucleic acid synthesis. Its activity is central to cellular proliferation. Knowledge about the mechanism of this pathway would be valuable in designing chemotherapeutic agents which can be used for the treatment and cure of leukaemia. This chapter presents the quantitative information on the rates of utilization of glucose and glutamine, and the metabolic interrelationships between them, in undifferentiated and DMSO-differentiated HL60 cells.

5.1 Materials and Methods

5.1.1 Chemicals and enzymes

All chemicals except radioisotopes were obtained from the Sigma Chemical Company, St. Louis, USA. The radioisotopes were from Amersham International, UK, and the enzymes and coenzymes were obtained from Boehringer Mannheim, Germany. The rest of the chemicals, media, inorganic solvents, etc. were obtained from the sources described in Section 2.1.1.

5.1.2 Synthesis of [U-^{14}C]-arabinose 5-phosphate

[U-^{14}C]-Arabinose 5-phosphate (0.5C_i/mol) was prepared from [U-^{14}C]-glucosamine 6-phosphate by the method of Volk (1966). [U-^{14}C]-Glucosamine 6-phosphate was first prepared from [U-^{14}C]-glucosamine at 30^{0}C as follows: the reaction mixture (2.0ml) contained [U-^{14}C]-glucosamine 6-phosphate (0.19µmol; 50 mCi); glycylglycine-KOH buffer, pH 7.4 (0.25mmol); ATP, (2.0µmol); $MgCl_2$, (4.0µmol); and hexokinase, (10 i.u.). After 30 mins the entire reaction mixture was applied to chromatography paper (Whatman 3MM, 40cm x 40cm), chromatographed in GW_3 solvent [butanol (320ml), n-

propanol (160ml), 80% HCOOH (200ml), 30% TCA (120ml) and acetone (200ml)] and run in the descending mode for 18 hours. The [U-^{14}C]-glucosamine 6-phosphate formed was located on the chromatogram with the help of authentic unlabelled glucosamine 6-phosphate, which was identified by the phosphate-detecting molybdate reagent (Rosenberg, 1959), as a marker. The [U-^{14}C]-glucosamine 6-phosphate, following elution from the paper, was added to 100 µmol of unlabelled glucosamine 6-phosphate in a total volume of 30 ml, which additionally contained ninhydrin (500µmol) and citrate buffer, pH 4.7, (0.75mmol), and the mixture was heated at 100^{0}C for 40 mins. The reaction mixture was cooled to 0^{0}C, filtered through Whatman No. 1 filter paper and applied to an ion-exchange column (1.5 x 30 cm) packed with Bio-Rad AG 1-X8 analytical grade resin (200-400 mesh) in the formate form. Elution was achieved using a linear gradient of ammonium formate (0.5M) and fractions ranging in size from 8-10ml were collected at a constant rate (0.8-1.0ml/min) using a fraction collector (Model 820, Instrument Specialties Co. Inc., USA) which maintained constant flow with a type 4912A pump (LKB, Sweden). Ammonium formate was removed from the appropriately pooled fractions by passing them through a column of Dowex 50W-X8 (200-400mesh, H^+ form resin). Elution was achieved with distilled water, and formic acid from the eluted sample was removed by liquid-liquid extraction with anhydrous ether for 18 hours. The [U-^{14}C]-arabinose 5-phosphate was further purified by paper chromatography using GW_3 solvent made up to 2% with phenyl boronic acid (Kapuscinski and Williams, 1984). The purified material was eluted from the chromatogram with distilled water. The aqueous solution obtained (pH 1-2) was further extracted with anhydrous ether to remove trichloroacetic acid. This preparation of [U-^{14}C]-arabinose 5-phosphate was shown to be free of ribose 5-phosphate, xylulose 5-phosphate and ribulose 5-phosphate by nuclear magnetic resonance and by gas liquid chromatographic analysis of the dephosphorylated, derivatized product (Arora, 1984).

5.1.3 Cells

Human promyelocytic HL60 cells were obtained, maintained and differentiated as described in Sections 2.1.2 and 2.1.3.

5.1.4 Incubation procedure for cells

Undifferentiated and DMSO-differentiated HL60 cells were collected and washed three times with phosphate-buffered saline at pH 7.4 (PBS). Incubations, which were performed at 37^0C in a shaking water bath at a cell density of 5×10^6 cells/ml, were initiated by the addition of suitable substrates. Preliminary studies showed that steady-state conditions, gauged from the linearity of the substrate utilization and product formation curves, prevailed during the 60 mins incubation. After 60 mins, the reactions were stopped by adding $HClO_4$(0.2ml of 4M) to each flask before placing them on ice. After centrifugation at 4^0C, the supernates were neutralized with a saturated solution of K_2CO_3 and, after further centrifugation at 4^oC to remove potassium perchlorate, were used for estimation of metabolites.

For the measurement of $^{14}CO_2$, cells were incubated at a concentration of 10^7 cells/ml in Warburg flasks (25ml) equipped with a centre well and a side arm. The centre well contained hyamine (1.0ml of 1.0 M) for trapping $^{14}CO_2$ and the side arm contained $HClO_4$ (0.2ml of 4M) for stopping the reaction. The reaction was initiated by the addition of the appropriate ^{14}C-labelled substrate (0.2μCi) in a final volume of 2ml so that the final concentrations of glucose and glutamine were 10mM and 3mM respectively. These concentrations were shown, in preliminary experiments, to be saturating for the individual substrates. Loaded flasks were placed in a shaking water bath for 30 mins at 37^0C. The reaction was stopped by adding $HClO_4$(0.2ml of 4M) from the side arm into the main flask compartment. The flasks remained in the shaking water bath for another 90 mins to allow complete absorption of $^{14}CO_2$. The ^{14}C radioactivity of the total hyamine solution (1.0ml) was measured by liquid scintillation counting. The scintillant fluid contained 6g of 2(4'-t-butylphenyl)-5-(4"-biphenyl)-1,3,4-oxadiazole (BDH Chemicals, UK) in toluene (600ml) and methoxyethanol (400ml). Scintillation counting was carried out using a Beckman LS-2800 Scintillation counter. All counting was corrected for quenching by the addition of a known volume of an external standard of n-[1-^{14}C] hexane to individual sample. The interference produced by the samples as a

result of quenching was evaluated by the known volume of the external standard itself and corrected for the final counts which was expressed as disintegration /min (dpm).

5.1.5 Enzyme preparation

With the exception of glutaminase, the maximum catalytic capacity of each of the selected enzymes was measured in a dialyzed 105,000 x g cell supernatant fraction (cytosol). For the preparation of cytosol, cells were collected, washed with Tris- HCl buffer (0.5M at pH 7.4), and then sonicated on ice, at a setting of 7, using an MSE 100 watt ultrasonic disintegrator for a total of 90 secs in three 30 sec pulses with an interval of 30 secs between each pulse. The sonicate was centrifuged at 10,000 x g for 10 mins at 4^0C. The supernate from this step was centrifuged at 105,000 x g in a Beckman Ultracentrifuge (Model TL-100) at 4^0C for one hour using the TL-100.2 rotor. The supernate was dialyzed for at least 12 hours against Tris- HCl buffer (0.05M at pH 7.4) at 4^0C. Separate experiments indicated that there was no loss of enzymic activity during dialysis. For the assay of glutaminase the complete cell sonicate was used.

5.1.6 Incubation procedure for the assay of non-oxidative pentose phosphate pathway

(a) Forward reaction: Utilization of ribose 5-phosphate for the production of hexose 6-phosphates and triose phosphates catalyzed by the cytosolic enzyme preparation:

The incubation mixture at 37^0C contained ribose 5-phosphate (24 μmol) and cytosolic enzyme preparation (5ml, equivalent to approximately 50 x 10^6 cells) to give a total volume of 7.4 ml at pH 7.4 (Arora,1984) . Samples (1.0ml) were removed from the incubation mixture at 0, 10, 20, 30, 40 and 60 mins and introduced into ice-cold $HClO_4$ (1.0 ml of 0.6M). The denatured protein was removed by centrifugation (5,000 x g for 10 mins) and the pH of the supernate adjusted to 6.8 with saturated $KHCO_3$. After removal of the precipitated $KClO_4$ by centrifugation at 4°C (10,000 x g for 10 mins) the final volume was adjusted to 3.0ml and samples were removed for analysis of the intermediates.

(b) Reverse reaction: Utilization of hexose 6-phosphate and triose phosphates to form ribose 5- phosphate by the cytosolic enzyme preparation:

The incubation mixture at 37^0C contained glucose 6-phosphate (17 µmole), fructose 1,6-diphosphate (28 µmole), cytosolic enzyme preparation (3ml, equivalent to approximately 50 x 10^6 cells) and distilled water to give a total volume of 5.5 ml at pH 7.4 (Arora,1984). Fructose 1,6-diphosphate was used as a source of triose phosphates. Samples (1.0ml) were removed from the incubation mixture at 0, 10, 20, 30, and 40 mins and introduced into ice-cold $HClO_4$ (1.0 ml of 0.6M). The denatured protein was removed by centrifugation (5,000 x g for 10 mins) and the pH of the supernate adjusted to 6.8 with saturated $KHCO_3$. After removal of the precipitated $KClO_4$ by centrifugation at 4^0C (10,000 x g for 10 mins) the final volume was adjusted to 3.0ml and samples were removed for analysis of the intermediates.

(c) Utilization of [U-^{14}C]-arabinose 5-phosphate for the production of hexose 6-phosphates and triose phosphates catalyzed by the cytosolic enzyme preparation:

The incubation mixture at 25^0C contained ribose 5-phosphate (42 µmol), [U-^{14}C]-arabinose 5-phosphate (8.4 µmol, 2 x 10^6 dpm) and cytosolic enzyme preparation (3ml, equivalent to approximately 10-15 x 10^6 cells) in a final volume of 5.0ml at pH 7.4 (Arora,1984). The reaction was initiated with the enzyme preparation and samples (2.0ml) were taken at 0 and 30 mins. A control reaction which used denatured protein (boiled for 5 mins) instead of the enzyme preparation accompanied the incubation. Samples were placed in a boiling water bath for 5 mins, cooled and centrifuged; residual protein in the supernatant fluid was removed by Millipore filtration (Cat no H AWP-025-00). Samples were lyophilized, made up to a final volume of 100µl and separated by paper chromatography using GW_3 solvent (made up to 2% with phenylboronic acid) run in a descending mode for 18 hours. The developed chromatogram was placed with Kodak X-O matt PP film (43 x 35 cm) for six weeks at -70^0C and the spots containing hexose monophosphates and arabinose 5-phosphate were detected by radiochromatography and counted by liquid scintillation as described in Section 5.1.4.

5.1.7 Metabolite assays

Metabolites present in neutralized cell extracts were measured spectrophotometrically using enzymic assays. Glucose was measured by the method of Bergmeyer *et al.* (1974), and the following metabolites were estimated by the methods given in parenthesis : glutamine and glutamate (Lund, 1974), lactate (Gutmann & Wahlefeld, 1974), pyruvate (Czok & Lamprecht, 1974), aspartate (Mollering, 1974), alanine (Williamson, 1974), glucose 6-phosphate (Lang & Michal, 1974), ribose 5-phosphate (Racker, 1974), pentoses (Blackmore and Williams, 1974), hexoses (Graham and Smydzuk, 1965) and fructose 1,6 diphosphate (Michal and Beutler, 1974). For the assay of glycogen, cells were collected, washed in PBS and sonicated; their glycogen content was measured using a modification, by Komuniecki & Saz (1982), of the method of Solling & Esmann (1975).

5.1.8 Assay of enzyme activities

Enzyme assays were carried out on fresh dialyzed cytosolic extracts or, in the case of glutaminase, on a fresh complete cell sonicate. Hexokinase, phosphofructokinase, pyruvate kinase and citrate synthase were measured according to Board *et al.* (1990), glutaminase by the method of Martin (1972), glycogen synthase according to Kim & Blatt (1969), NADP-linked malic enzyme by the method of Rutter & Lardy (1958), glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase by the method of Glock & McLean (1953), transketolase and transaldolase by the method of Clark *et al.* (1972) and aldolase by the method of Rajkumar *et al.* (1966).

5.2 Results

5.2.1 Glucose metabolism

Both types of HL60 cells utilized glucose as sole substrate at a similar rate of 30nmol/min/10^7 cells (Table 5). A major proportion (73%) of the glucose utilized by undifferentiated HL60 cells was converted to lactate and this rose marginally to 83% after 9 days of DMSO treatment. With both types of cells, the production of $^{14}CO_2$ from [U-

Table 5: Glucose and glutamine metabolism in HL60 and DMSO-treated cells.

Cell Type	Substrate used	Glucose	Lactate+Pyruvate	Lactate/Pyruvate	Glutamine	Glutamate	Aspartate	Alanine
HL60	None	-	3.3±0.64			3.60±0.31	0.87±0.112	0.41±0.00
	Glucose(10mM)	-30±2.30	47.7±5.60	12.8	ND	4.10+0.70	ND	ND
	Glutamine(3mM)	ND	3.3±0.64		-4.6±0.33	1.50±0.15	1.16±0.03	0.26±0.00
	Glucose(10mM) + Glutamine(3mM)	-17±0.88[a]	32.0±1.44	9.6	-9.0±0.83[c]	0.84±0.20	0.50±0.06	0.21±0.00
DMSO-differentiated HL60	None	-	3.7±0.47			3.08±0.22	0.90±0.13	0.50±0.00
	Glucose(10mM)	-30±2.66	53.6±5.38	10.8	ND	3.46±1.09	ND	ND
	Glutamine(3mM)	ND	3.7±0.47		-8.0±0.15	2.50±0.31	0.41±0.00	0.10±0.00
	Glucose(10mM) + Glutamine(3mM)	-21±1.60[b]	29.5±1.97	6.1	-10.0±0.55	1.16±0.18	0.17±0.02	0.12±0.00

Cells were incubated as described in the Materials and Methods section in the presence of substrate(s) as indicated. Values are expressed as nmol/10^7cells/min. Each value is the mean ± S.E.M. of at least three separate experiments, each performed in duplicate. (-) indicates utilization; ND means not determined. a and b are significantly different from the cells incubated with glucose alone ($p<0.01$ and $p<0.05$ respectively); and c is significantly different compared with the cells incubated with glutamine alone ($p<0.01$).

^{14}C]-glucose accounted for only 2% of the glucose consumed (Table 5.1) and the maximum catalytic activities of the glycolytic enzymes (hexokinase, phosphofructokinase, pyruvate kinase) and citrate synthase remained unchanged after DMSO treatment (Table 5.3). However, the maximum catalytic activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, the key regulatory enzymes of the oxidative segment of the pentose phosphate pathway, were approximately three to six times higher in the DMSO-differentiated cells (Table 5.3). The increased activities of these two enzymes is consistent with the selectively higher rate of $^{14}CO_2$ formation from [1-^{14}C] compared with [6-^{14}C]-glucose, which led to an increase in the C_1/C_6 ratio of two-fold in favour of the DMSO-treated cells (Table 5.2). In contrast, the maximum catalytic activities of the enzymes of the non-oxidative pentose phosphate pathway (aldolase, transketolase and transaldolase) remained unchanged after DMSO-differentiation (Table 5.3). The stability of these reactions is consistent with the unchanged reaction rates (both forward and reverse) of the non-oxidative pentose phosphate pathway as assessed by the 'Stop assay' which provides a quantitative assessment of the maximum catalytic capacity of the entire pathway (Arora, 1984). The 'Stop assay' showed (Figs 5 to 5.5) that the rate of ribose 5-phosphate utilization was rapid compared with the rates at which hexose 6-phosphate and triose phosphate accumulated (Figs 5 and 5.1). The rate of ribose 5-phosphate utilization was not linear over the entire period of incubation and two widely-divergent rates were observed: the maximum rate was rapid (18nmol/min/10^6) during the initial 5 min of reaction for both cell types and it declined to a slower rate of approximately 4 nmol/min /10^6 cells during the next 20 mins (Figs 5 and 5.1; Table 5.4). In spite of the identical pattern of ribose 5-phosphate utilization, the rates at which hexose 6-phosphate and triose phosphate accumulated were approximately two-fold higher in the DMSO-differentiated cells (Table 5.4). In both cell types, however, hexose 6-phosphate and triose phosphate were formed in a 2:1 ratio, which is in general agreement with the stoichoimetry of the pentose pathway reaction sequence. The 'Stop assay' for the reverse reactions of the pentose phosphate pathway confirmed the presence of these reactions operating at equal rates in

Table 5.1: [U-^{14}C] glucose and [U-^{14}C] glutamine conversion to $^{14}CO_2$ by HL60 and DMSO-treated cells.

Cell Type	Substrate used	[U-^{14}C] Glucose to $^{14}CO_2$	[U-^{14}C] Glutamine to $^{14}CO_2$
HL60	Glucose(10mM)	0.69±0.02	-
	Glutamine(3mM)	-	1.53±0.13
	Glucose(10mM) + Glutamine(3mM)	0.59±0.03	2.23±0.05
DMSO-differentiated HL60	Glucose(10mM)	0.69±0.14	-
	Glutamine(3mM)	-	2.76±0.31
	Glucose(10mM) + Glutamine(3mM)	0.39±0.07	1.20±0.12

Cells were incubated as described in the Materials and Methods section in the presence of [U^{14}C]-glucose or [U^{14}C]-glutamine as indicated. Values are given as nmol/10^7cells/min and are means ± S.E.M. of at least three separate experiments, each performed in triplicate. ND means not determined. $^{14}CO_2$ production was calculated from the total concentration of glucose (10mM) or glutamine (3mM) (radioactive + normal) added to the incubation mixture having counts of approximately 4 x 10^5 dpm (0.2μCi), in a total volume of 2ml, containing 2 x 10^7 cells. The reaction rate was measured for 30 mins and values are expressed as nmol of glucose or glutamine being converted to $^{14}CO_2$ /10^7 cells/min.

Table 5.2: Evolution of $^{14}CO_2$ from [1-^{14}C] and [6-^{14}C] glucose by HL60 and DMSO-treated cells.

Cell Type	HL60		DMSO-differentiated HL60	
Glucose(10mM)	[1-^{14}C] glucose	[6-^{14}C] glucose	[1-^{14}C] glucose	[6-^{14}C]glucose
$^{14}CO_2$ production	2.02± 0.09	0.57± 0.03	1.92±0.42	0.28±0.01
C_1/C_6	3.42±0.08[a]		6.80±1.26	
F-type PC (%)	<0.01		<0.01	
Glucose(10mM)+Glutamine(3mM)				
$^{14}CO_2$ production	1.24± 0.08	0.16± 0.02	1.13±0.06	0.18±0.03
C_1/C_6	7.5±1.10[b]		6.35±1.25	

Cells were incubated in the presence of 10mM glucose and ± 3mM glutamine as described in the Materials and Methods section. All values are expressed as nmole/10^7 cells/min and are means ± S.E.M. of at least three separate experiments, each performed in duplicate. a is significantly different from DMSO-treated cells ($p<0.06$); while b is significantly different compared to cells incubated with glucose alone ($p<0.05$). $^{14}CO_2$ production was calculated from the total concentration of glucose (10mM) (radioactive + normal) added to the incubation mixture having counts of approximately 4 x 10^5 dpm (0.2μCi), in a total volume of 2ml containing 2 x 10^7 cells. The reaction rate was measured for 30 mins and values are expressed as nmol of glucose being converted to $^{14}CO_2/10^7$ cells/min.

Table 5.3: Maximum catalytic activities of marker enzymes of glycolysis, glutaminolysis and pentose-phosphate pathways in HL60 and DMSO-treated cells.

Enzyme activities U/10^7 cells	HL60	DMSO-differentiated cells
Pyruvate Kinase	490 ± 40.0	496 ± 20.0
Phosphofructokinase	37 ± 4.6	31 ± 4.0
Hexokinase	32 ± 0.5	26 ± 4.0
Citrate Synthase	99 ± 3.8	91 ± 2.0
Glucose 6-phosphate dehydrogenase	5 ± 0.3	25 ± 1.0[a]
6-Phosphogluconate dehydrogenase	26 ± 4.3	66 ± 8.0[b]
Glycogen Synthase	2 ± 0.3	0
Glutaminase	6 ± 0.7	3 ± 0.5[c]
NAD(P)-linked Malic Enzyme	24 ± 0.2	22 ± 1.0
Aldolase	19 ± 0.1	16 ± 1.0
Transketolase	1 ± 0.1	1 ± 0.2
Transaldolase	1 ± 0.1	1 ± 0.1

Cells were prepared, cytosolic extracts made and enzymes assayed as described in the Materials and Methods section. **U** (Units of enzyme measured, one unit of enzyme activity is defined as the formation of one nanomole of product per minute).

All data are the means ± S.E.M. of three separate experiments, each performed in triplicate. a, b and c are significantly different from HL60 cells ($p<0.02$).

Fig 5: Ribose 5-phosphate utilization and the production of hexose and triose-phosphates catalyzed by cytosolic enzyme preparation from HL60 cells.

The incubation mixture at 37^0C contained ribose 5-phosphate (24 μmol) and cytosolic enzyme preparation (5ml, equivalent to approximately 50 x 10^6 cells) to give a total volume of 7.4 ml at pH 7.4. Samples (1.0ml) were removed from the incubation mixture at 0, 10, 20, 30, 40 and 60 mins and introduced into ice-cold $HClO_4$ (1.0 ml of 0.6M). The denatured protein was removed by centrifugation (5,000 x g for 10 mins) and the pH of the supernate adjusted to 6.8 with saturated $KHCO_3$. After removal of the precipitated $KClO_4$ by centrifugation at 4^0C (10,000 x g for 10 mins), the final volume was adjusted to 3.0ml and samples were removed for analysis of the intermediates. All values are the means ± S.E.M. of three different experiments, each performed in duplicate.

(5) Ribose 5-phosphate utilization and the production of hexose and triose-phosphates catalyzed by cytosolic enzyme preparation from HL60 cells

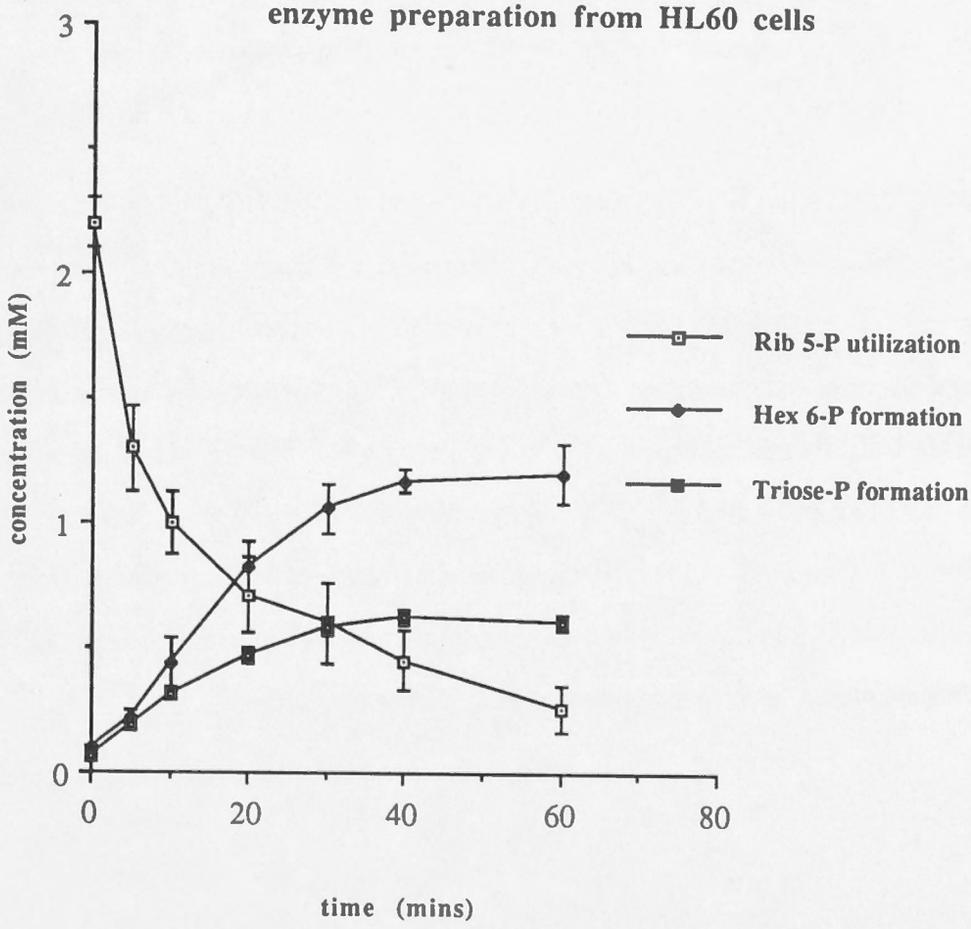


Fig 5.1: Ribose 5-phosphate utilization and the production of hexose and triose-phosphates catalyzed by cytosolic enzyme preparation from 9 day DMSO-treated cells.

The incubation mixture at 37^0C contained ribose 5-phosphate (24 μmol) and cytosolic enzyme preparation (5ml, equivalent to approximately 50 x 10^6 cells) to give a total volume of 7.4 ml at pH 7.4. Samples (1.0ml) were removed from the incubation mixture at 0, 10, 20, 30, 40 and 60 mins and introduced into ice-cold $HClO_4$ (1.0 ml of 0.6M). The denatured protein was removed by centrifugation (5,000 x g for 10 mins) and the pH of the supernate adjusted to 6.8 with saturated $KHCO_3$. After removal of the precipitated $KClO_4$ by centrifugation at 4^0C (10,000 x g for 10 mins), the final volume was adjusted to 3.0ml and samples were removed for analysis of the intermediates. All values are the means $\pm$ S.E.M. of three different experiments, each performed in duplicate.

(5.1) Ribose 5-phosphate utilization and the production of hexose and triose phosphates catalyzed by cytosolic enzyme preparation from 9 day DMSO-treated cells

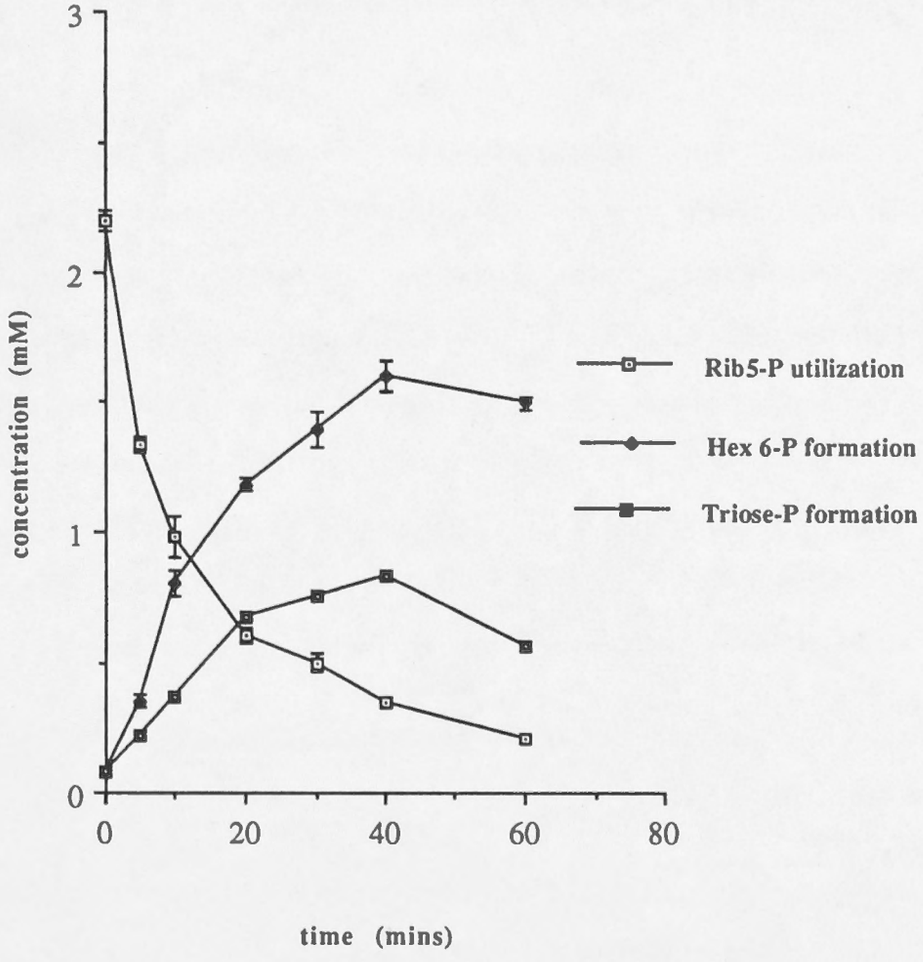


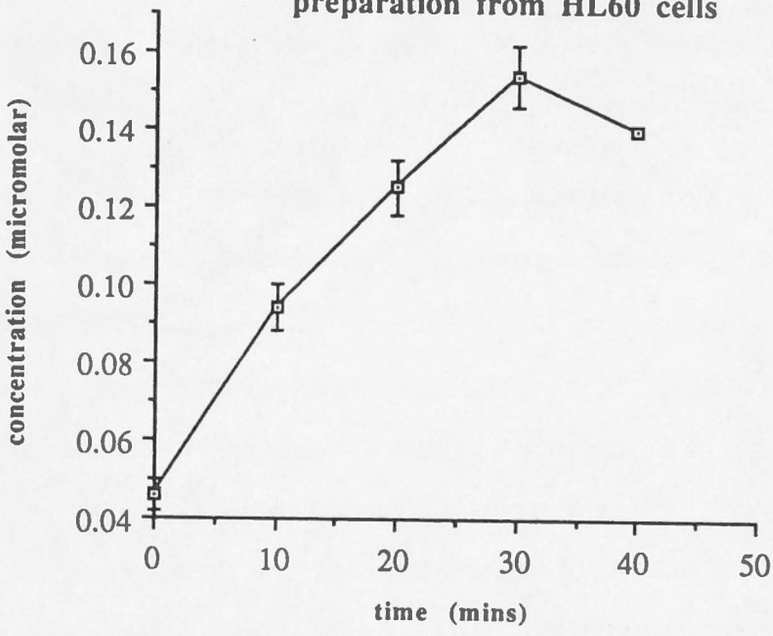
Fig 5.2: Ribose 5-phosphate production from hexose 6-phosphate and triose phosphate by the cysolic preparation from HL60 cells.

The incubation mixture at 37^0C contained glucose 6-phosphate (17 μmole), fructose 1,6-diphosphate (28 μmole), cytosolic enzyme preparation (3ml, equivalent to approximately 50 x 10^6 cells) and distilled water to give a total volume of 5.5 ml at pH 7.4. Fructose 1,6-diphosphate was used as a source of triose phosphates. Samples (1.0ml) were removed from the incubation mixture at 0, 10, 20, 30, and 40 mins and introduced into ice-cold $HClO_4$ (1.0 ml of 0.6M). The denatured protein was removed by centrifugation (5,000 x g for 10 mins) and the pH of the supernate adjusted to 6.8 with saturated $KHCO_3$. After removal of the precipitated $KClO_4$ by centrifugation at 4^0C (10,000 x g for 10 mins), the final volume was adjusted to 3.0ml and samples were removed for analysis of the intermediates. All values are the means ± S.E.M. of three different experiments, each performed in duplicate.

Fig 5.3: Hexose 6-phosphate and triose phosphate utilization by the cytosolic preparation from HL60 cells.

The conditions for the experiment are the same as those described in Fig 5.2. All values are the means ± S.E.M. of three different experiments, each performed in duplicate.

(5.2) Ribose 5-phosphate production from hexose 6-phosphate and triose-phosphate by the cytosolic preparation from HL60 cells



(5.3) Hexose 6-phosphate and triose-phosphate utilization catalyzed by the cytosolic preparation from HL60 cells

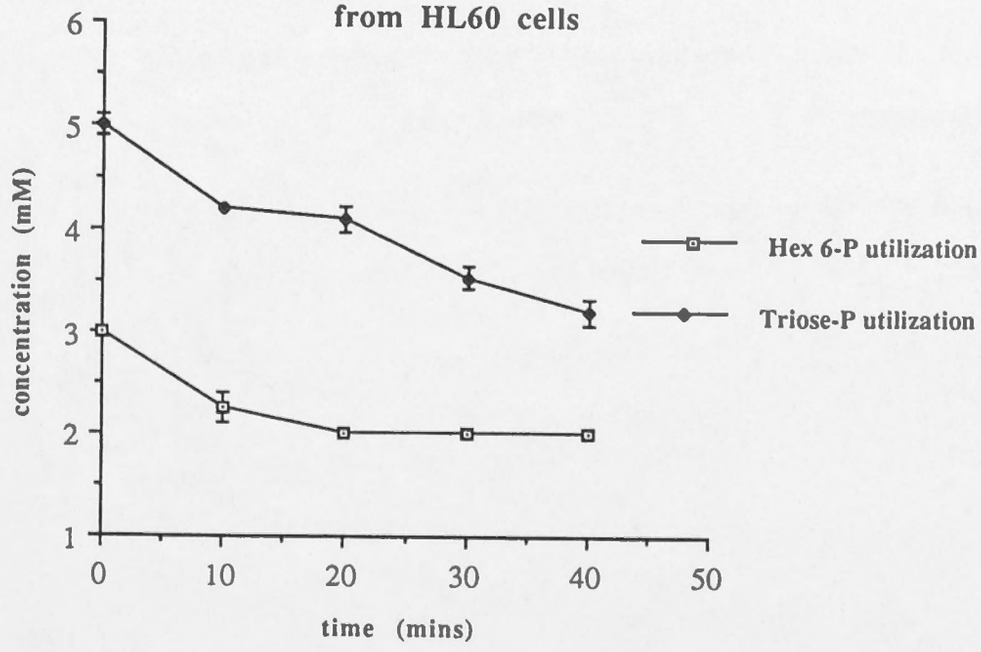


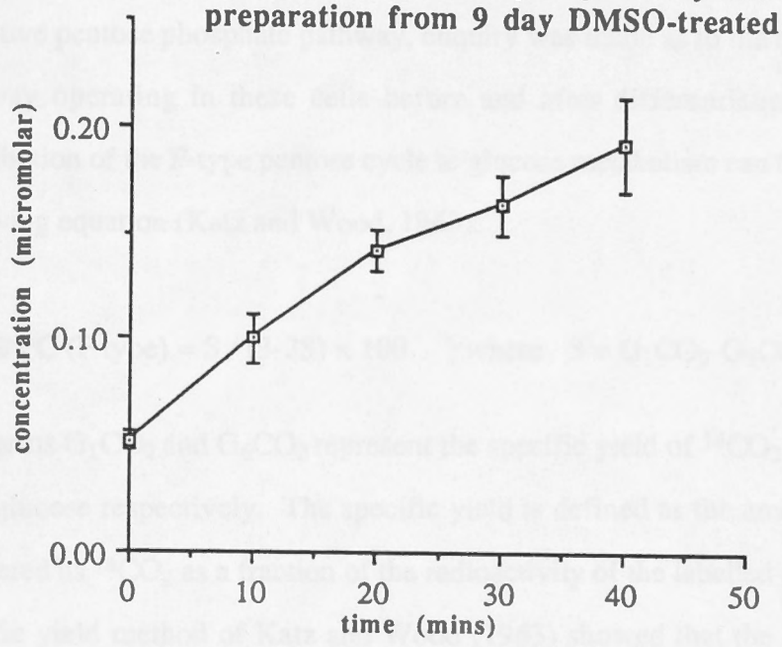
Fig 5.4: Ribose 5-phosphate production from hexose 6-phosphate and triose phosphate by the cysolic preparation from 9 day DMSO-treated cells.

The incubation mixture at 37^0C contained glucose 6-phosphate (17 μmole), fructose 1,6-diphosphate (28 μmole), cytosolic enzyme preparation (3ml, equivalent to approximately 50 x 10^6 cells) and distilled water to give a total volume of 5.5 ml at pH 7.4. Fructose 1,6-diphosphate was used as a source of triose phosphates. Samples (1.0ml) were removed from the incubation mixture at 0, 10, 20, 30, and 40 mins and introduced into ice-cold $HClO_4$ (1.0 ml of 0.6M). The denatured protein was removed by centrifugation (5,000 x g for 10 mins) and the pH of the supernate adjusted to 6.8 with saturated $KHCO_3$. After removal of the precipitated $KClO_4$ by centrifugation at 4^0C (10,000 x g for 10 mins), the final volume was adjusted to 3.0ml and samples were removed for analysis of the intermediates. All values are the means ± S.E.M. of three different experiments, each performed in duplicate.

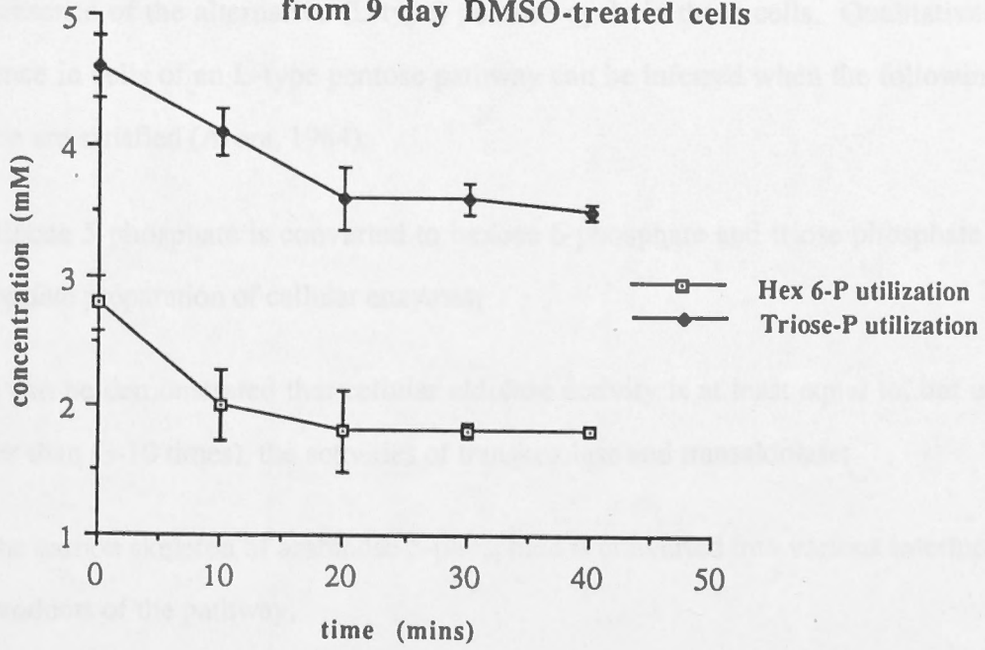
Fig 5.5: Hexose 6-phosphate and triose phosphate utilization by the cytosolic preparation from 9 day DMSO-treated cells.

The conditions for the experiment are the same as those described in Fig 5.3. All values are the means ± S.E.M. of three different experiments, each performed in duplicate

(5.4) Ribose 5-phosphate production from hexose 6-phosphate and triose phosphate by the cytosolic preparation from 9 day DMSO-treated cells



(5.5) Hexose 6-phosphate and triose-phosphate utilization catalyzed by cytosolic preparation from 9 day DMSO-treated cells



the differentiated and undifferentiated cells which were, however, several-fold lower than those of the forward reactions (Table 5.4).

Having established the presence of both forward and reverse reactions of the non-oxidative pentose phosphate pathway, enquiry was made as to the *type* of non-oxidative pathway operating in these cells before and after differentiation. The quantitative contribution of the F-type pentose cycle to glucose metabolism can be estimated using the following equation (Katz and Wood, 1963):

$$\% \text{ PC (F type)} = S / (3\text{-}2S) \times 100 \quad ; \text{ where } \quad S = G_1CO_2\text{-}G_6CO_2 / 1\text{-} G_6CO_2$$

The terms G_1CO_2 and G_6CO_2 represent the specific yield of $^{14}CO_2$ from [1-^{14}C] and [6-^{14}C]-glucose respectively. The specific yield is defined as the amount of [^{14}C]-isotope recovered as $^{14}CO_2$ as a fraction of the radioactivity of the labelled glucose utilized. The specific yield method of Katz and Wood (1963) showed that the F-type pentose cycle apparently contributes less than 0.01% to the total glucose metabolism of both HL60 cells and their DMSO-differentiated counterparts (Table 5.2). Hence, evidence was sought for the presence of the alternative (L-type) pentose cycle in these cells. Qualitatively, the presence in cells of an L-type pentose pathway can be inferred when the following four criteria are satisfied (Arora, 1984):

(1) Ribose 5-phosphate is converted to hexose 6-phosphate and triose phosphate by an appropriate preparation of cellular enzymes;

(2) It can be demonstrated that cellular aldolase activity is at least equal to, but usually greater than (3-10 times), the activities of transketolase and transaldolase;

(3) The carbon skeleton of arabinose 5-phosphate is converted into various intermediates and products of the pathway;

(4) D-glycero-D-ido octulose 1,8-diphosphate is formed and converted into hexose 6-phosphates by the cellular enzyme preparation.

Table 5.4 (a) : Rates of utilization of pentose 5-phosphate together with the rate of hexose 6-phosphate and triose phosphate production by cytosolic enzyme preparations from HL60 and 9 day DMSO-treated cells.

	Pentose 5-phosphate			
	A. Ribose 5-phosphate		Hexose 6-phosphate	Triose phosphate
	0-5 mins	5-20 mins	0-10 mins	0-10 mins
HL60	-18.8	-3.8	3.5	1.24
9 day DMSO-treated cells	-17.2	-4.6	7.9	2.96
	B. Ribose 5-phosphate+ [U-^{14}C]-Arabinose 5-phosphate		Hexose 6-phosphate	Triose phosphate
	0-30 mins		0-30 mins	
HL60	-0.42		0.27	ND
9 day DMSO-treated cells	-0.64		0.60	ND

All rates are expressed as nmol/min/10^6 cells. In the case of B (ribose 5-phosphate + [U-^{14}C]-arabinose 5-phosphate), only the utilization of [U-^{14}C]-arabinose 5-phosphate is shown. In the case of B, hexose 6-phosphate production was from total pentoses (ribose 5-phosphate + [U-^{14}C]-arabinose 5-phosphate). (-) indicates utilization; ND means not determined.

Table 5.4 (b): Rates of hexose 6-phosphate and triose phosphate utilization together with the rate of ribose 5-phosphate production by the cytosolic enzyme preparations of HL60 and 9 day DMSO-treated cells.

Cell type	Hexose 6-phosphate	Triose phosphate	Ribose 5-phosphate
	0-10 mins	0-10 mins	0-20 mins
HL60	-8.88	-6.0	0.55
9 day DMSO-treated cells	-9.40	-9.4	0.56

The rates of hexose 6-phosphate and triose phosphate utilization are expressed as nmol/min/10^6 cells while the rate of ribose 5-phosphate production is expressed as pmol/min/10^6 cells. (-) indicates utilization.

Of the above four criteria, (1) and (2) have been discussed above and the presence of the reactions was demonstrated by the 'Stop assay' method. The measurement of aldolase activity showed that it was several-fold higher than that of transaldolase and transketolase in both cell types (Table 5.3). Criterion number (3) was also measured by the 'Stop assay' method, which involved the use of 1 part of [U-^{14}C]-arabinose 5-phosphate and 9 parts of unlabelled ribose 5-phosphate. [U-^{14}C]-Arabinose 5-phosphate was utilized by the cytosolic enzyme preparation from both cell types and converted to hexose 6-phosphates. The concentration of hexose 6-phosphates formed, however, was much lower than that observed when ribose 5-phosphate was used alone (Table 5.4a), perhaps because of the inhibition of transaldolase by arabinose 5-phosphate (Arora, 1984). This experiment suggests that arabinose 5-phosphate is an intermediate of the non-oxidative pentose phosphate pathway that can be converted to hexose 6-phosphates in both types of cells. Hence, the presence of an L-type pentose phosphate pathway can be inferred in HL60 and DMSO-differentiated cells, but the results are not sufficient to make any conclusive quantitative statement on the status of this pathway. Criterion number (4) was not followed in this thesis and awaits further investigation.

The uptake and utilization of glucose was inhibited significantly by added glutamine in both types of cells, with the effect being more pronounced in HL60 cells (45%) than in the DMSO-treated cells (30%). The inhibition of glucose utilization by glutamine was accompanied by a two-fold increase in the steady-state concentration of glucose 6-phosphate and ribose 5-phosphate in the undifferentiated HL60 cells (Tables 5.5 and 5.6). These observations are consistent with the decreased production of $^{14}CO_2$ from [1-^{14}C] and [6-^{14}C]-glucose in the presence of glutamine in both cell types and the enhancement of the C_1/C_6 ratio in the case of HL60 cells (Table 5.2). The accumulation of glucose 6-phosphate in the presence of glutamine may also be responsible for the six-fold higher content of glycogen in HL60 cells (Table 5.5) since the long-term culture medium that these cells are maintained in contains both glucose and glutamine (Section 2.1). The higher glycogen content in HL60 cells agrees with the significant activity of

Table 5.5: Ribose 5-phosphate and glycogen content of HL60 and DMSO-treated cells.

Cell Type	Ribose 5-Phosphate (nmol/10^7 cells)	Glycogen (μg /10^7 cells)
HL60	11 ± 0.9	43.7 ± 2.0
DMSO-differentiated cells	4 ± 0.9^a	6.8 ± 0.8^b

Ribose 5-phosphate and the glycogen content in the cells were measured as described in the Materials and Methods. All data are the means $\pm$ S.E.M. of three separate experiments, each performed in triplicate. a and b are significantly different from HL60 cells ($p<0.01$).

Table 5.6: Accumulation of glucose 6-phosphate and change in the glycogen concentration induced by glucose and glutamine substrates in undifferentiated HL60 cells.

Cell Type	Substrates used	Glucose 6-phosphate (nmol/10^7cells)	Change in glycogen content above the basal level (μg/10^7cells)
HL60	Glucose(10mM)	11.8±0.8	0.00
	Glucose(10mM) + Glutamine(3mM)	22.0±0.4[a]	2.8±0.15

Cells were incubated as described in the Materials and Methods section in the presence of substrate(s) as indicated. All data are the means ± S.E.M. of three separate experiments, each performed in triplicate. a is significantly different in comparison with that from cells incubated with glucose ($p<0.01$).

glycogen synthase found in these cells compared with the undetectably low activity in the DMSO-differentiated cells (Table 5.3).

5.2.2 Glutamine metabolism

When glutamine was used as a sole substrate, it was utilized by undifferentiated HL60 cells at approximately half the rate of DMSO-treated cells in spite of a two-fold higher catalytic activity of glutaminase in the undifferentiated cells (Table 5.3). A significant proportion (32%) of the glutamine consumed by both types of cells was recovered as glutamate and no lactate was found. In undifferentiated cells, 25% of the glutamine accumulated as aspartate and 6% as alanine, while this proportion fell to 5% aspartate and 2% alanine upon differentiation (Table 5). Significant percentages of glutamine utilized were oxidized to $^{14}CO_2$ (35%) (Table 5.1). When glucose was added to the incubation medium glutamine utilization was enhanced, with the effect being more noticeable in the undifferentiated HL60 cells. A very high proportion (94%) of the glucose consumed in the presence of glutamine was converted to lactate in the HL60 cells compared to 70% in the case of DMSO-treated cells (Table 5). The accumulation of glutamate and aspartate was reduced by added glucose by a factor of two to three-fold in both cell types (Table 5) compared with the situation when glutamine was used as the sole substrate. The lower accumulation of glutamate and aspartate in the case of the undifferentiated cells may be attributed to the high rate of oxidation of glutamine via the NAD(P)-linked malic enzyme pathway (Fig 1.8), which is consistent with the enhanced oxidation of [U-^{14}C]-glutamine to $^{14}CO_2$ (Table 5.1). On the other hand, in DMSO-treated cells, enhanced utilization of glutamine in the presence of glucose was followed by decreased accumulation of aspartate and glutamate, and decreased oxidation of [U-^{14}C]-glutamine to $^{14}CO_2$. More pyruvate accumulated in these cells (Table 5) than in their undifferentiated counterparts, possibly because of the lower (or inhibited) activities of appropriate mitochondrial enzymes required for the metabolism of intramitochondrial pyruvate.

5.3 Discussion

5.3.1 Glucose metabolism

Suspensions of undifferentiated and DMSO-differentiated HL60 cells both utilized glucose as sole substrate at a similar rate (Table 5). This rate was considerably higher than that reported for macrophages (Newsholme *et al.*, 1986), Ehrlich ascites tumour cells (Medina *et al.*, 1988), rat muscle cells (Challiss *et al.*, 1986) and rat brain cells (Hawkins *et al.*, 1971). The maximum catalytic activity of hexokinase, which represents the point of entry of intracellular glucose into dissimilative pathways, was sufficient to accommodate 88-100% of the glucose utilized by the differentiated and undifferentiated cells respectively. The activities of hexokinase, phosphofructokinase, pyruvate kinase and citrate synthase, which were all within the range reported for tumour cells from other sources, were the same before and after differentiation (Board *et al.*, 1990). Thus, any difference in the glycolytic behaviour of these cells is likely to have been due to the short-term regulation of the pathway rather than to adaptive changes in the maximum catalytic capacity of its constituent enzymes via enzyme synthesis. The proportion of the glucose taken up, but not glycolyzed, by both cell types may include some incorporation into glycogen, the concentration of which was six-fold higher in the undifferentiated HL60 cells (Table 5.5). The higher glycogen content is consistent with the significant activity of glycogen synthase in undifferentiated cells and the lack of detectable activity of this enzyme in the DMSO-treated cells.

The maximum catalytic activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were approximately three to six times higher in the DMSO-treated cells (Table 5.3). The lower activity of glucose 6-phosphate dehydrogenase in the undifferentiated cells may restrict the entry of glucose into the reactions of the oxidative segment of the pentose phosphate pathway (OPP). This difference in enzyme capacity is reflected in the rates of $^{14}CO_2$ formation from [1-^{14}C] and [6-^{14}C]-glucose: values of 3.4 and 6.8 were obtained for the C_1/C_6 ratio (the ratio of the specific radioactivity of $^{14}CO_2$ arising from [1-^{14}C]-glucose compared with that from

[6-^{14}C]-glucose oxidation) (Williams *et al.*, 1987) in the undifferentiated and DMSO-differentiated HL60 cells respectively (Table 5.2). Although the absolute value of this ratio provides only qualitative information, the values reported in Table 5.2 are compatible with significant OPP activity in HL60 cells which becomes more fully activated following DMSO-differentiation. This behaviour may permit the maintenance of the NADP/ NADPH couple in a highly reduced state compatible with the synthesis and delivery of reduced oxygen species by cells that share some of the functional and phenotypic characteristics of neutrophils (Ahmed *et al.*, 1991).

The maximum catalytic activities of the enzymes of the non-oxidative pentose phosphate pathway were unaltered upon differentiation (Table 5.3). This is consistent with the same overall flux of ribose 5-phosphate, hexose 6-phosphate and triose phosphate through the forward and reverse reactions of the non-oxidative limb of the pentose phosphate pathway in both cell types (Table 5.4 and Figs 5-5.5). The two-fold greater accumulation of hexose 6-phosphate and triose phosphate by the cytosolic preparation from DMSO-treated cells, in spite of the same net amount of ribose 5-phosphate utilized by the undifferentiated HL60 cells, is not easily explained. Since the dialyzed cytosolic cellular preparations were free of added nucleotides (unless they were covalently bound to enzymes) there should be no reactions of glycolysis, gluconeogenesis, etc. to cause net removal of hexoses and triose phosphates by conversion to other products. Moreover, the metabolic versatility of the 'Stop assay' system was also limited by the absence of added ATP; this is a essential component of the PRPP synthetase reaction whose expressed activity, if included in the incubation medium, could alter the rate and extent of formation of pentose phosphate pathway intermediates and products. The presence of endogenous ATP could, however, divert the flux of ribose 5-phosphate towards the synthesis of PRPP in rapidly-proliferating HL60 cells (which have approximately six-fold more PRPP synthetase activity, Chapter 6), thereby decreasing the steady-state concentrations of hexose 6-phosphates and triose phosphates compared to the values measured in DMSO-treated cells. Measurement of the F-type pentose phosphate pathway showed that it made a negligible contribution (< 0.01%) to glucose metabolism in these

cells. Experiments with [U-^{14}C]-arabinose 5-phosphate, demonstration of the conversion of ribose 5-phosphate to hexose 6-phosphate and triose phosphates (Table 5.4) and confirmation of the presence of aldolase with activity several-fold higher than that of transketolase and transaldolase in both cell types, indicated that an alternative (L-type) non-oxidative pathway might be present. There is insufficient evidence to allow the contribution of this pathway to glucose metabolism to be defined quantitatively.

When incubated with glucose alone, 73% of the glucose consumed was converted to lactate in HL60 cells and this proportion rose only marginally (to 83%) upon differentiation. The production of $^{14}CO_2$ from [U-^{14}C]-glucose accounted for only about 2% of the total glucose consumed by both cell types, indicating that the complete oxidation of the pyruvate derived from glucose is not a significant energy source. The small number of mitochondria revealed by electron microscopy (Chapter 2) or a very low activity of pyruvate transport or pyruvate dehydrogenase might account for the restricted oxidation of glycolytically-generated pyruvate when this is a potential source of acetyl CoA. Since undifferentiated HL60 cells are derived from promyelocytic cells blocked at an early stage of differentiation, and DMSO-differentiation converts them to a neutrophil-like morphology, the mitochondrial number in differentiated cells is not expected to be high. Moreover, in rapidly-proliferating neoplastic cells, mitochondrial pyruvate dehydrogenase may be partially inhibited by the production of superoxide formed from oxygen (Eigenbrodt *et al.*, 1985). In addition, the extramitochondrial pyruvate generated from glucose via the glycolytic pathway may not be transported efficiently to mitochondrial pyruvate dehydrogenase and hence is reduced to cytoplasmic lactate by lactate dehydrogenase according to the prevailing steady-state cytoplasmic NAD/NADH ratio (Dworkin and Dworkin-Rastl, 1991). As a result, the high accumulation of lactate observed in the presence of glucose in case of both undifferentiated and differentiated cells may be attributed to the partial/complete inhibition of pyruvate transport or pyruvate dehydrogenase.

The uptake and utilization of glucose was inhibited significantly by added glutamine in both cell types, although the effect was more marked in the undifferentiated (45%) than

the DMSO-differentiated cells (30%) (Table 5). If total glucose utilization is related, in part, to the activity of phosphofructokinase, as it is in many tissues, an effect of glutamine on glucose disappearance may be more noticeable in the undifferentiated cells, which have a lower activity of the alternative (OPP) metabolic route. Inhibition of phosphofructokinase, with the resultant accumulation of hexose 6-phosphates, may lead to the inhibition of glucose utilization at the point of its primary phosphorylation. The two-fold increase in the steady-state concentration of glucose 6-phosphate observed upon the addition of glutamine is compatible with the inhibition of glycolysis at the phosphofructokinase locus (Table 5.6). However, some coordinated inhibition of glucose uptake and flux through the OPP may also be expected if hexokinase is inhibited by ribose 5-phosphate or some other intermediate of the pentose phosphate pathway which accumulates. The concentration of ribose 5-phosphate was higher by a factor of two in the undifferentiated cells (Table 5.5). Inhibition of hexokinase by ribose 5-phosphate has been reported for Ehrlich ascites tumour cells (Gumaa and McLean, 1969) and a similar phenomenon may be operative in HL60 cells. These observations are further supported by the inhibition of $^{14}CO_2$ production from both [1-^{14}C] and [6-^{14}C]-glucose in the presence of glutamine (Table 5.2). Glutamine, however, enhanced the C_1/C_6 ratio two-fold in undifferentiated cells, suggesting that a greater flow of glucose carbon through the OPP may be stimulated by glutamine to provide biosynthetic intermediates for the synthesis of nucleic acids in the rapidly-proliferating cells. In contrast, glutamine had no effect on the C_1/C_6 ratio in the differentiated cells.

Significant inhibition of glucose utilization by glutamine (up to 25%) has been reported in colonocytes (Ardawi and Newsholme, 1985) as a consequence of the inhibition of phosphofructokinase. In human diploid fibroblasts (Zielke *et al.*, 1975), the presence of glutamine stimulated [1-^{14}C]-glucose oxidation and simultaneously inhibited [6-^{14}C]-glucose oxidation; such re-routing of glucose carbon may restrict the provision of additional pentose phosphate pathway intermediates for purine and pyrimidine synthesis (via PRPP) to those situations where sufficient glutamine is available. These effects of glutamine on glucose utilization are in contrast to those reported for enterocytes and

lymphocytes (Watford *et al.*, 1979; Ardawi and Newsholme, 1983) in which glutamine increased the utilization of glucose by *enhancing* the activity of phosphofructokinase. In Ehrlich ascites tumour cells (Medina *et al.*, 1988) glutamine had no significant effect on glucose metabolism.

5.3.2 Glutamine metabolism

When glutamine was the sole substrate, it was utilized by undifferentiated HL60 cells at approximately half the rate of that of the differentiated cells (Table 5), despite a two-fold difference in the total catalytic capacity of glutaminase in favour of the undifferentiated cells (Table 4). Intracellular glutamine transport rather than glutaminase may be the factor which limits glutamine utilization in the undifferentiated cells. Glutamine transport into the mitochondrial matrix is a carrier-mediated process that is dependent on the intracellular concentration of Na^+ and the energy status of the cells (Kovacevic & McGivan, 1984). In renal cortex mitochondria, cytoplasmic acidosis increases the activity of the glutamine carrier by increasing either the number of carrier sites or the rate of transport by existing carriers (Simpson *et al.* , 1975). In undifferentiated HL60 cells, the rate of glutamine transport across the mitochondrial membrane may be limited by the low intrinsic activity of the Na^+/H^+ exchanger at the plasma membrane (Besterman and Cautrecasas, 1984). Differentiation induced by DMSO has been shown to activate this exchanger in HL60 cells (Restrepo *et al.*, 1987). The greater influx of Na^+ may enhance the access of glutamine to glutaminase by increasing the rate of transport of glutamine across the mitochondrial inner membrane. Differentiated cells may therefore have a greater overall rate of glutamine utilization in spite of their intrinsically lower glutaminase activity. In both cell types, the OPP may become operative when glucose is added to the glutamine-containing medium, as a result of which the acid equivalents produced would tend to lower the cytoplasmic pH (Borregaard *et al.*, 1984). A compensatory activation of the Na^+/H^+ exchanger, which extrudes protons from the cytoplasm in exchange for an influx of Na^+, would enhance, in turn, the transport of glutamine within the cell and provide access to mitochondrial glutaminase (Simpson *et al.*, 1975). Glutamine utilization is enhanced by glucose in both cell types, but the percentage increase is greater in the

undifferentiated HL60 cells owing to their lower basal rate of glutamine consumption. The effect of glucose on the glutaminolysis of HL60 cells contrasts with that reported for other tumour cell lines in which the rate of glutaminolysis is responsive to conditions which lead to the inhibition of P_i-dependent glutaminase (Eigenbrodt *et al.*, 1985).

In incubations with glutamine alone, approximately 30% of the glutamine consumed was recovered as glutamate in both cell types and no lactate was formed. In the undifferentiated cells, 25% of the glutamine accumulated as aspartate and 6% as alanine, while these proportions fell to 5% aspartate and 2% alanine upon differentiation. These differences may reflect a higher rate of synthesis of aspartate, compatible with its utilization for the synthesis of purines and pyrimidines, in the rapidly-proliferating undifferentiated cells, which declines as the activity of aspartate amino-transferase falls during differentiation. Significant amounts of the glutamine utilized were oxidized to $^{14}CO_2$ (35%) in both cell types (Tables 5 and 5.1). The oxidation of glutamine to CO_2 may be attributed to the presence of mitochondrial NAD(P)-linked malic enzyme. It has been postulated that this isoenzyme is responsible for the formation of pyruvate from glutamine in tumour cell mitochondria (Sauer *et al.*, 1980; Moredith and Lehninger, 1984). The activity of this enzyme in embryo cells *in vitro* is sufficient to account for all of the pyruvate formed by these cells (Dworkin and Dworkin-Rastl, 1989; Lϕvtrup-Rein and Nelson, 1982). Since the metabolic state of many mammalian tumour cells (Pedersen, 1978; Eigenbrodt et al., 1985) is similar in several respects to the enzymology of embryo cells, the significance of pyruvate formation from mitochondrial glutamate (produced by cytoplasmic glutamine via mitochondrial membrane-bound glutaminase) through mitochondrial NAD(P)-linked malic enzyme and its further oxidation by mitochondrial pyruvate dehydrogenase (Fig 1.8) may be to provide energy and precursors to support high rates of cell division. Hence, in rapidly-proliferating neoplastic cells, NAD(P)-linked malic enzyme is ideally located to be an essential component of glutamine metabolism as a source of aerobically-generated ATP as well as serving as a regulatory enzyme capable of generating precursors at a sufficient rate to meet the anabolic demands of neoplastic cells.

One consequence of glutamine addition in the presence of glucose was an increase in the steady-state concentration of hexose-monophosphates, manifested as a two-fold increase in glucose 6-phosphate concentration (Table 5.6). Glucose 6-phosphate, besides being a glycolytic intermediate, is also available for glycogen synthesis and pentose phosphate formation so that the elevation of its level makes the cells functionally ready for biosynthetic or other metabolic demands. This is in agreement with the results presented in Tables 5.2 and 5.6 which show that the presence of both glucose and glutamine accelerated the relative flux through the pentose phosphate pathway (C_1/C_6 ratio increased from 3.8 to 7.5) and also triggered a small net synthesis of glycogen. In undifferentiated cells the decreased accumulation of aspartate and glutamate in the presence of both glutamine and glucose occurred possibly as a result of the increased net utilization of these amino acids for the synthesis of purines and pyrimidines when both substrates were present. This observation is consistent with the enhanced oxidation of [U-^{14}C]-glutamine to $^{14}CO_2$. On the other hand, in DMSO-differentiated cells, under similar conditions, decreased accumulation of aspartate and glutamate was accompanied by a reduced rate of oxidation of [U-^{14}C]-glutamine to $^{14}CO_2$ (Tables 5 and 5.1) and a higher steady-state concentration of pyruvate (as seen by a lower lactate/pyruvate ratio), possibly as a result of reduced activities of mitochondrial pyruvate-utilizing enzymes. The pyruvate concentration did not increase to the same extent in the undifferentiated HL60 cells (Table 5).

The present study emphasizes that neoplastic cells oxidize exogenous glutamine for energy demands and also to provide precursors for anabolic purposes. In both neoplastic and DMSO-differentiated cells exogenous glucose is converted mainly to lactate. Differentiation of HL60 cells by DMSO had no major effects on the overall rates of glucose utilization and lactate formation, even though the pathways of glucose metabolism had a different emphasis. In both cell types the net utilization of glucose and glutamine appeared to be regulated reciprocally. Although the pathway for glutamine metabolism was not affected in a major way by differentiation, there was a significant shift in the pattern of glucose metabolism. DMSO-differentiated HL60 cells metabolized

glucose in the same fashion as phagocytic cells (Newsholme *et al.*, 1986) showing that, with respect to glucose metabolism, their differentiation had given a much greater emphasis to the NADPH-generating reactions of the pentose phosphate pathway. These cells may thus be used to mimic myeloid differentiation in experiments in which the behaviour of undifferentiated cells is compared with an appropriate control.

Chapter 6: Purine and pyrimidine metabolism in HL60 and DMSO-treated cells

6: Introduction

The alterations in metabolism that occur during the development of normal cells follow a complex program of selective gene activation and repression. In the genesis of leukaemic cells this orderly program is disrupted and the sequential changes in gene expression that characterize normal development are perturbed. HL60 cells have provided a means of investigating the biochemical events that accompany the experimentally-induced maturation of leukaemic cells *in vitro* and, by implication, of assessing the extent to which these changes are related to those regulating gene expression during normal cell development. Whilst the maturation of HL60 cells appears to have been arrested at the early promyelocytic stage, these cells can be stimulated by DMSO *in vitro* to differentiate along the granulocytic lineage and thereby to acquire certain morphological and functional characteristics of mature neutrophils (Collins, 1987; Chapters 2 and 3). Purine and pyrimidine nucleotide metabolism play an important role in the provision of precursors for nucleic acid synthesis during cellular maturation. Studies with different leukaemic cell lines have shown that cellular maturation can be promoted by incubation with purine bases or purine analogues (hypoxanthine, 6-thioguanine and 6-mercaptopurine) which inhibit nucleotide biosynthesis (Lucas *et al.*, 1983; Nguyen *et al.*, 1984; Ishiguro and Sartorelli, 1985). Increased activities of enzymes of purine and pyrimidine nucleotide biosynthesis have been observed in rat hepatomas that grow at different rates and in other animal and human neoplasms (Weber *et al.*, 1983; Jackson *et al.*, 1980). These observations are consistent with the appearance of increased concentrations of nucleotides and deoxynucleotides that are linked to neoplastic 'transformation and progression' (Weber, 1983). It has also been observed that lymphoblastic and lymphocytic leukaemia cells express abnormally high activities of enzymes that regulate the metabolism of purine nucleotides (Smyth *et al.*, 1978; Scholar and Calabresi, 1973).

Aim of the study

The present chapter describes features of the purine nucleotide pathway in undifferentiated and DMSO-differentiated HL60 cells that distinguish them from PMNs isolated from human blood. Malignant cells because of their proliferative nature are known to over-produce purine and pyrimidine nucleotides (Weber *et al.*, 1982 and Weber,1983). Hence, a detailed knowledge of the regulation of purine metabolism in normal leukocytes and their leukaemic counterparts is important in elucidating critical biochemical differences that may be exploited in chemotherapy and/or in defining the metabolic basis of antileukaemic drug resistance.

In the present study purine and pyrimidine nucleotide metabolism in both untreated cultured HL60 cells and those induced to mature with DMSO were examined. Changes in the rate of purine and pyrimidine nucleotide biosynthesis as well as in the activities of the enzymes of both the *de novo* and salvage pathways of purine nucleotide biosynthesis were evaluated (Sections 6.1.4-6.1.8). The status of purine and pyrimidine nucleotide biosynthesis in undifferentiated and differentiated cells was assessed as follows: The *de novo* purine nucleotide pathway was evaluated by the incorporation of [^{14}C]-formate and the salvage pathway by the incorporation of [^{14}C]-hypoxanthine (Sections 6.1.4 and 6.1.5). Pyrimidine nucleotide biosynthesis was measured by the incorporation of $NaH^{14}CO_3$ (Section 6.1.6). The simultaneous estimation of the rates of both purine and pyrimidine nucleotide synthesis *de novo* was also determined (Section 6.1.6). The later method is important in permitting the influence of a number of metabolites on the simultaneous activities of purine and pyrimidine nucleotide synthesis. The availability of PRPP is crucial to cell metabolism. The activity of pentose phosphate pathway and the intracellular concentration of ribose 5-phosphate are powerful regulators of PRPP formation. Neoplastic and transformed cells have very high concentrations of PRPP (Sochor *et al.*, 1989). Hence, the concentration of important metabolites like ribose 5-phosphate, PRPP and IMP (which determines the relative synthesis of adenylates and guanylates) was determined (Section 6.1.9) and their effects on purine nucleotide biosynthesis were evaluated in both undifferentiated and differentiated cells. Since it is

believed that the rates of purine and pyrimidine nucleotide biosynthesis are directly dependent on ribose 5-phosphate and PRPP synthesis from glucose and that a decreased concentration of adenylates and guanylates leads to increased flux through the pentose phosphate pathway (Sochor *et al.*, 1989), experiments were designed to show whether the activities of purine and pyrimidine pathways are wholly dependent on pentose phosphate pathway activity. To that end, experiments were done using phenazine methosulphate and the contribution of oxidative pentose phosphate pathway to glucose metabolism was assessed using C_1/C_6 ratio (Section 6.1.3). The effect of phenazine methosulphate on PRPP production and hence purine nucleotide biosynthesis was also assessed (Sections 6.1.4 and 6.1.9). These experiments would provide useful information in showing how ribose 5-phosphate production in leukaemic and differentiated cells is regulated by the oxidative pentose phosphate pathway to meet the cell's need for nucleotide biosynthesis. These experiments would also provide information to show how events which lead to differentiation of these cells impinge on the functions of pentose phosphate pathway and the pathways of purine and pyrimidine metabolism.

The above investigation will not only provide valuable information to gauge the consequences of differentiation from neoplastic to the differentiated state but will also establish the chemical background against which nucleic acid and consequent cellular protein synthesis occur.

6.1 Materials and Methods

6.1.1 Chemicals and enzymes

All chemicals except radioisotopes were obtained from the Sigma Chemical Company, St. Louis, USA. The radioisotopes were from Amersham International, UK and New England Nuclear, USA. The enzymes were obtained from Boehringer Mannheim, Germany. Polyethyleneimine-impregnated thin layer plates were from Merck, USA.

6.1.2 Cells

Human promyelocytic HL60 cells were obtained, maintained and differentiated as described in Sections 2.1.2 and 2.1.3. Human PMNs from a healthy adult female subject were separated by the methods described in Section 2.1.5.

6.1.3 Assay of the oxidative segment of pentose phosphate pathway in the presence of phenazine methosulphate

The contribution of the oxidative segment of the pentose phosphate pathway to glucose metabolism in the presence of phenazine methosulphate (0.1mM) was determined by assessment of the C_1/C_6 ratio as described in Section 5.1.4.

6.1.4 Assay of the rate of *de novo* purine nucleotide synthesis

Undifferentiated and DMSO-differentiated HL60 cells were collected and washed with phosphate-buffered saline (PBS) at pH 7.4. The rate of purine nucleotide synthesis *de novo* was assayed according to the method of Hershfield and Seegmiller (1976) with slight modifications. An aliquot of cells (10^6 cells/ml) was incubated under 5% CO_2 and 95% air at 37^0C in the presence of 10% dialyzed foetal calf serum, 10mM glucose $\pm$ 3mM glutamine, and $\pm$ 0.1mM phenazine methosulphate in a final volume of 1.0ml. The cells were pulsed with [^{14}C]-formate (320 nmole, 51mCi/mmole) for 40 mins. The pulse was terminated by placing the tubes on ice for 5 mins (longer periods on ice, up to one hour, had no effect on the results), centrifuging at 300 x g at 4^0C for 5 mins, collecting the medium from above the cell pellet (*i.e.* the extracellular fluid), and replacing it with $HClO_4$ (2.0 ml of 0.4M). After mixing, the assay for total intracellular purines was continued by incubating the perchloric acid-treated cells at 100^0C for one hour, chilling to 0^0C and centrifuging at 800 x g for 10 mins. The supernate (containing the free purine bases derived from all intracellular purine-containing soluble nucleotides and polynucleotides) was then applied to a column of Dowex 50-H^+ (100 to 200 mesh, 2 x 0.4 cm) and washed with HCl (5ml of 0.1N) before eluting the purine bases as a single fraction with more concentrated HCl (5 ml of 6N). A sample (1.0ml) of this solution

was counted in 10ml of 'Aquasol' to determine the rate of labelling *de novo* of total intracellular purines. The remainder was evaporated under reduced pressure at 40^0C, the residue dissolved in water (200 µl) and a sample (50 µl) applied to polyethyleneimine-impregnated thin layer plates and chromatographed in a single dimension in n-butanol: acetone: glacial acetic acid: ammonia solution (5%): water (35:25:15:15:10) using adenine, guanine and hypoxanthine as markers. These three compounds were identified under ultraviolet light, cut out, counted, and the counts in each fraction corrected for the total counts recovered in the 6N HCl fraction. More than 90-95% of the counts in the 6N HCl fraction were in adenine, guanine and hypoxanthine. The labelling of intracellular purines was linear for at least 45 mins at the concentration of [^{14}C]-formate used.

The total extracellular purines released into the medium were assayed by treating the collected medium (the extracellular fluid) with $HClO_4$ (1.0ml of 0.4M). After mixing, the assay was continued by incubating the perchloric acid-treated media at 100^0C for one hour, and the rest of the separation and analysis was performed as described in the previous paragraph.

In some experiments (see Tables 4 and 5) the rates of labelling of acid-soluble and insoluble nucleotides were determined separately by initially extracting the cell pellet twice with $HClO_4$ (1.0ml of 0.4M) at 0^0C for 15 mins. The extracts were combined and $HClO_4$ (2 ml of 0.4M) added to the pellet. Both the acid-soluble extracts and the nucleic acid-containing pellets were hydrolyzed and chromatographed separately as described for the separation of intra and extracellular purines/purine nucleotides. For determination of the counts in RNA and DNA purines, the extracted pellet was hydrolyzed overnight in NaOH (0.5 ml of 0.3N) at 37^0 C and neutralized with HCl. Calf thymus DNA (50 µg) and $HClO_4$ (2 ml of 0.4M) were added. After centrifuging at 4^0C for 20 mins at 1000 x g, the supernates containing ribonucleotides were removed and the DNA-containing pellets resuspended in $HClO_4$(2 ml of 0.4M). Both were then hydrolyzed and processed as for the whole cells.

6.1.5 Assay of the rate of salvage purine nucleotide synthesis

For the assay of the salvage pathway, the method used for the *de novo* pathway was followed except that the cells were pulsed with [^{14}C]-hypoxanthine instead of [^{14}C]-formate.

6.1.6 Assay of the rate of *de novo* purine and pyrimidine nucleotide synthesis

Undifferentiated and DMSO-differentiated HL60 cells were collected and washed with phosphate-buffered saline at pH 7.4 (PBS). The rates of both purine and pyrimidine nucleotide synthesis *de novo* were assayed according to the method of Huisman *et al.* (1979) with slight modifications. Cells were incubated at a concentration of 1 x 10^6/ml in Warburg flasks (25ml) equipped with a centre well containing hyamine (1.0 ml of 1.0 M) for trapping $^{14}CO_2$, and a side arm containing $HClO_4$ (0.2ml of 4M) for stopping the reaction. The reaction was initiated by the addition of ^{14}C-labelled $NaHCO_3$ (300nmole, 58μCi/μmol) in a final volume of 2ml so that the final concentrations of glucose, glutamine, $NaHCO_3$ and foetal calf serum were 10mM, 3mM, 10mM and 10% respectively. Loaded flasks were placed in a shaking water bath and incubated for one hour at 37^0C. The reaction was stopped by tipping $HClO_4$ (0.2ml of 4M) from the side arm into the main compartment. The flasks remained in the shaking water bath for another 90 mins to allow complete absorption of $^{14}CO_2$. The ^{14}C radioactivity of the total hyamine solution (1.0ml) was measured by liquid scintillation counting as described in Section 5.1.4. The acidified samples in the main compartments of the Warburg flasks were collected and incubated at 100^0C for one hour, chilled at 0^0C for 10 mins and centrifuged at 800 x g for 10 mins. The supernates (containing the free purine and pyrimidine bases derived from all intracellular purine and pyrimidine-containing soluble nucleotides and polynucleotides) were applied to a column of Dowex 50-H^+ (100 to 200 mesh, 2 x 0.4 cm). Pyrimidine bases were eluted as a single fraction with HCl (4ml of 0.1N) before the purine bases were eluted (also as a single fraction) with more concentrated HCl (4 ml of 6N). Samples (1.0ml) of both pyrimidine and purine-

containing solutions were counted in 10ml of 'Aquasol' to determine the rate of labelling *de novo* of total intracellular pyrimidines and purines. The method employed for isolation of pyrimidine and purine compounds permited the determination of label in: (1) all pyrimidine compounds and their precursors except carbamoyl phosphate; and (2) in all stable purine compounds distal to the incorporation of bicarbonate into the pathway of purine nucleotide synthesis *de novo*.

6.1.7 Enzyme preparation

The maximum catalytic activities of the enzymes were measured in a 30,000 x g extract prepared from sonicated washed cells. Cells were sonicated for 90 secs on ice, at a setting of 7, using an MSE 100 watt ultrasonic disintegrator that delivered three 30 sec pulses with an interval of 30 secs between each pulse. The sonicate obtained was centrifuged at 30,000 x g for 45 mins at 4^0C in a Beckman Ultacentrifuge (Model TL-100). The supernate obtained from this step was used for the enzymic assays. For the assay of 5′-nucleotidase, the method of Camici *et al.* (1990) was followed. Membrane fractions were prepared by dissolving the pellet from the 30,000 x g step in the same volume of PBS containing Triton X-100 (2%). The suspension was stirred for 90 mins at 37^0C to solubilize or release the membrane-bound proteins, and then centrifuged at 30,000 x g for 45 mins at 4^0C. The supernate was used for the assay of 5′-nucleotidase.

6.1.8 Assay of enzyme activities

Enzyme assays were carried out on fresh cytosolic extracts. PRPP synthetase was measured by the method of Kunjara *et al.* (1986), amidophosphoribosyl transferase by the method of Martin (1972), IMP dehydrogenase by the method of Jackson *et al.* (1977), adenylosuccinate synthetase by the method of Lieberman (1956), hypoxanthine/guanine phosphoribosyl transferase (HGPRT), adenine phosphoribosyl transferase (APRT), nucleoside phosphorylase, adenosine deaminase and 5′-nucleotidase according to the method of Camici *et al.* (1990).

6.1.9 Metabolite Assays

Inosine monophosphate (IMP) was measured by the method of Grassl (1974) and PRPP by a modification of the method of Henderson and Khoo (1965). The assay for PRPP involves the release of $^{14}CO_2$ from [^{14}C-carboxyl]-orotic acid in the presence of the enzymes oritidine-5 phosphate pyrophosphorylase and oritidine-5 phosphate decarboxylase. $^{14}CO_2$ released is trapped by hyamine solution and counted by liquid scintillation counting. The assay is based on the following reactions:

[^{14}C-carboxyl]-orotic acid + PRPP + Mg^{+2} → [^{14}C-carboxyl] oritidine 5-phosphate → Uridine 5-phosphate + $^{14}CO_2$ (trapped by hyamine)

The first reaction occurs in the presence of the enzyme oritidine-5 phosphate pyrophosphorylase while the second one is initiated by oritidine-5 phosphate decarboxylase.

For the measurement of PRPP, cells at a concentration of 20 x 10^6/ml were incubated for 30 mins at 37^{0}C in the presence of 10mM glucose ±3mM glutamine and 10% dialyzed foetal calf serum. The reaction was stopped by heating the suspension in a boiling water bath for 30 secs and then chilling on ice for a further 15 mins. An aliquot (2ml) of the PRPP-containing supernatant (prepared by centrifuging at 10,000 x g for 15 mins) was loaded into a 25 ml Warburg flask (as described previously) containing unlabelled orotic acid (2µmole), [^{14}C-carboxyl]-orotic acid (1µCi) and $MgCl_2$ (2µmole). The reaction was initiated by adding a mixture of oritidine-5 phosphate pyrophosphorylase and oritidine-5 phosphate decarboxylase (0.5U of each). Loaded flasks were incubated in a shaking water bath at 37^{0}C for 90 mins. The reaction was stopped by adding $HClO_4$ (0.2ml of 4M) from the side arm into the main compartment. The flasks were kept in the shaking water bath for another 90 mins to allow complete absorption of $^{14}CO_2$. The ^{14}C-radioactivity of the total hyamine solution present in the centre well of the Warburg flasks (1.0ml) was measured by liquid scintillation counting as described in Section 5.1.4.

6.2 Results

6.2.1 Synthesis of PRPP

Although the maximum catalytic activity of PRPP synthetase was three-fold higher in the undifferentiated cells (Table 6), this was not matched by an increase in the steady-state concentration of PRPP, which remained the same in both cell types (Table 6.1). Addition of phenazine methosulphate (0.1mM) to the incubation medium increased the activity of the oxidative segment of the pentose phosphate pathway, as indicated by an eight-fold increase in the C_1/C_6 ratio in the undifferentiated cells and a three-fold increase in the differentiated cells (Fig 6). There were matching increases in the concentration of PRPP in the undifferentiated and differentiated cells respectively (Table 6.1). The presence of glutamine in the incubation medium increased the concentration of PRPP only slightly (by a factor of 1.2 to 1.5) in both cell types.

6.2.2 Synthesis of purine nucleotides by the *de novo* pathway

Whilst both undifferentiated and differentiated HL60 cells incorporated [^{14}C]-formate into purine nucleotides, demonstrating that *de novo* purine nucleotide synthesis occurred in these cells, the rate of incorporation was six-fold higher in the undifferentiated cells (Table 6.2). DMSO-induced differentiation caused a fourteen-fold and an eleven-fold decrease in the labelling of the intracellular guanosine and adenosine nucleotide pools respectively (Tables 6.3 & 6.4). At the same time, incorporation of [^{14}C]-formate was lowered by twelve-fold in the soluble fraction and three-fold in the DNA fraction while the labelling of the RNA fraction remained unchanged. Table 6.2 shows that about 44% of the purine nucleotides synthesized by the undifferentiated cells were released into the medium [mostly as hypoxanthine (58%)] (Table 6.3), while DMSO-differentiated cells released 38% of the total purine nucleotides synthesized [predominantly as adenine (55%)] (Table 6.4). The maximum catalytic activities of two of the putative rate-limiting enzymes of the *de novo* pathway were depressed significantly in the differentiated cells: amidophosphoribosyl transferase and adenylosuccinate synthetase were both depressed

Table 6 : Maximum catalytic activities of key regulatory enzymes of purine metabolism in HL60 and DMSO-differentiated cells.

Enzyme activity (U/10^6 cells)	HL60	DMSO-differentiated cells	PMNs
PRPP synthetase	0.23±0.01	0.07±0.00[a]	0.02±0.01[e]
Amidophosphoribosyl transferase	0.36±0.00	0.12±0.01[b]	0.07±0.00[f]
IMP dehydrogenase	0.25±0.01	0.19±0.02[c]	ND
Adenylosuccinate synthetase	0.39±0.02	0.07±0.01[d]	ND
HGPRT	0.44±0.02	0.30±0.00	0.09±0.02[g]
APRT	0.23±0.01	0.20±0.00	0.03±0.01[h]
NP	6.92±0.64	6.16±0.86	ND
AD	0.93±0.13	0.94±0.12	ND
5′Nucleotidase	33.00±2.20	39.00±4.30	ND

Enzymes were extracted and assayed as described in the Materials and Methods section. U (Units of enzyme measured, one unit of enzyme is defined as one nanomole of product formed per minute, except in the case of 5′-nucleotidase where one unit of enzyme is defined as one μg of P_i liberated per 2.5 hour). All data represent the means ± S.E.M. of three separate experiments, each performed in triplicate. a,b,c,d,e,f,g and h are significantly different from HL60 cells ($p < 0.01$).

Table 6.1: PRPP and IMP levels in HL60 and DMSO-differentiated cells.

Cell Type	Additives	PRPP(nmol/10^7 cells)	IMP(nmol/10^7 cells)
Undifferentiated HL60 cells	glucose (10mM)+glutamine(3mM)	0.4±0.02[a]	154±6.4
Undifferentiated HL60 cells	glucose(10mM)+glutamine(3mM)+phenazine methosulphate(0.1mM)	2.3±0.17	ND
Undifferentiated HL60 cells	glucose (10mM)	0.3±0.01	ND
DMSO-differentiated cells	glucose (10mM)+glutamine(3mM)	0.4±0.02[b]	84±12[c]
DMSO-differentiated cells	glucose(10mM)+glutamine(3mM)+phenazine methosulphate(0.1mM)	1.5±0.07	ND
DMSO-differentiated cells	glucose (10mM)	0.3±0.02	ND

The cells were incubated with the respective additives for 30 mins and metabolites were extracted and assayed as described in the Materials and Methods section. All data represent the means ± S.E.M. of at least three different experiments, each performed in triplicate. a and b are significantly different compared to cells incubated in the presence of 0.1mM PMS ($p < 0.01$) and c is significantly different from HL60 cells ($p < 0.01$).

Fig 6: C_1/C_6 ratio in HL60 and DMSO-differentiated cells.

1, **2** and **3** are undifferentiated HL60 cells incubated in the presence of 10mM glucose, 10mM glucose+ 3mM glutamine, and 10mM glucose+ 3mM glutamine+ 0.1mM phenazine methosulphate respectively. **4**, **5** and **6** are the DMSO-differentiated cells incubated under conditions similar to **1**, **2** and **3**. The cells were incubated as described in the Materials and Methods section. The values are expressed as C_1/C_6 ratio and are means $\pm$ S.E.M. of at least three separate experiments, each performed in duplicate. a is significantly different compared to the undifferentiated HL60 cells incubated in the presence of 10mM glucose only ($p<0.06$); b is significantly different (in the case of HL60 cells) compared with cells incubated in the presence of glucose only ($p<0.05$); c and d are significantly different compared with cells (c, in the case of HL60 cells, and d for DMSO-differentiated cells) incubated in the absence of phenazine methosulphate ($p<0.01$).

(6) C1/C6 ratio in HL60 and DMSO-differentiated HL60 cells

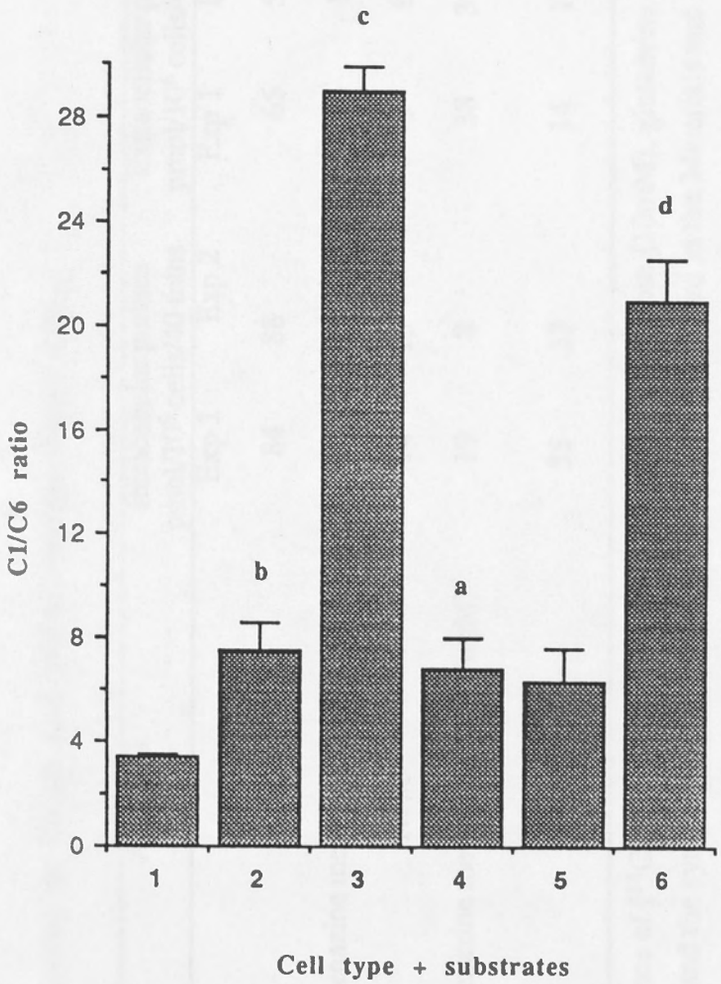


Table 6.2: [^{14}C]-Formate labelling in HL60 and DMSO-differentiated cells.

Cell Type	Additives	Intracellular purines pmol/10^6 cells/40 mins		Extracellular purines pmol/10^6 cells/40 mins		Total purines pmol/10^6 cells/40 mins	
		Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
Undifferentiated HL60 cells	None	84	88	65	55	149	143
Undifferentiated HL60 cells	Phenazine methosulphate (0.1mM)	82	60	135	120	217	180
DMSO-differentiated cells	None	14	13	9	9	23	22
DMSO-differentiated cells	Phenazine methosulphate (0.1mM)	10	8	38	30	48	38
PMNs	None	35	33	14	12	49	45

Cells were incubated in the presence of [^{14}C]-formate (320nmole, 51mCi/mmole), glucose (10mM), glutamine (3mM) and dialyzed foetal calf serum (10%) for 40 minutes and the synthesis of purines were evaluated as described in the Materials and Methods section. Data are from two separate experiments, each performed in duplicate.

Table 6.3: Labelling of intracellular purines with [^{14}C]-formate by undifferentiated HL60 cells.

Purine bases	Soluble fraction (1)	RNA (2)	DNA (3)	Sum (1+2+3)	Whole cell	Medium
Adenine	53.08	2.06	6.06	61.2	61.54	22.51
Guanine	19.00	1.27	1.23	21.5	17.57	5.09
Hypoxanthine	3.4	2.57	0.212	6.18	5.02	38.16
All purines	75.48	5.9	7.5	88.88	84.13	65.76

A culture of HL60 cells at 1 x 10^6/ml in the presence of 10mM glucose, 3mM glutamine and 10% dialyzed foetal calf serum was labelled with [^{14}C]-formate (320 nmole, 51μCi/μmole). After 40 minutes, two 2-ml aliquots were removed for analysis. The cells in one aliquot were fractionated to determine separately isotopic labelling of purine bases in soluble, RNA and DNA nucleotides. The cells in the second aliquot were hydrolyzed without prior fractionation for determination of labelling of total intracellular purines directly. All values are expressed as picomole of bases/10^6 cells/40 mins and each is the mean of two incubations performed in duplicate. The variation between the data from two experiments was in the range of 5-10%.

Table 6.4: Labelling of intracellular purines with [^{14}C]-formate by DMSO-differentiated cells.

Purine bases	Soluble fraction (1)	RNA (2)	DNA (3)	Sum (1+2+3)	Whole cell	Medium
Adenine	1.32	2.45	0.529	4.29	5.5	5.04
Guanine	2.32	1.65	0.635	4.6	1.26	2.02
Hypoxanthine	2.75	0.952	1.11	4.8	7.26	2.08
All purines	6.39	5.05	2.27	13.7	14.69	9.14

A culture of DMSO treated HL60 cells at 10^6/ml in the presence of 10mM glucose, 3mM glutamine and 10% dialyzed foetal calf serum was labelled with [^{14}C]-formate (320 nmole, 51μCi/μmole). After 40 minutes, two 2-ml aliquots were removed for analysis. The cells in one aliquot were fractionated to determine separately isotopic labelling of purine bases in soluble, RNA and DNA nucleotides. The cells in the second aliquot were hydrolyzed without prior fractionation for determination of labelling of total intracellular purines directly. All values are expressed as picomole of bases/10^6 cells/40 mins and is the mean of two incubations performed in duplicate. The variation between the data from two experiments was in the range of 5-10%.

in activity by three to five-fold while the activity of IMP dehydrogenase decreased only marginally (Table 6).

6.2.3 Synthesis of purine nucleotides by the salvage pathway

[^{14}C]-Hypoxanthine incorporation demonstrated that the purine salvage pathway was active in HL60 cells. The undifferentiated HL60 cells incorporated hypoxanthine at double the rate of the DMSO-differentiated cells, even though the rates of synthesis of adenine and guanine nucleotides were the same in both cell types (Table 6.5). The maximum activities of HGPRT, APRT, nucleoside phosphorylase (NP), adenosine deaminase (AD) and 5′-nucleotidase were unaffected by DMSO-induced differentiation (Table 6).

6.2.4 Effect of phenazine methosulphate on purine nucleotide synthesis (*de novo* and salvage pathways)

Phenazine methosulphate enhanced the total synthesis of purine nucleotides by the *de novo* pathway (approximately two-fold) in both cell types. The enhancement was detected only in the concentration of the extracellular purine nucleotides (Table 6.2). Phenazine methosulphate slightly enhanced the activity of the salvage pathway in the differentiated cells, and in fact eliminated the original two-fold difference in the uptake of hypoxanthine which had favoured the undifferentiated cells (Table 6.5).

6.2.5 *De novo* and salvage pathways in polymorphonuclear leukocytes (PMNs)

In human peripheral blood PMNs, purine nucleotide synthesis by the *de novo* pathway was only 30% of that of the undifferentiated cells but still twice that of the DMSO-differentiated cells (Tables 6.2 and 6.6). The activities of PRPP synthetase and amidophosphoribosyl transferase, however, were significantly lower in PMNs than in the differentiated cells (Table 6). The activity of the salvage pathway in PMNs was five times lower than that in both differentiated and undifferentiated HL60 cells, which is

Table 6.5: [^{14}C]-Hypoxanthine labelling in HL60 and DMSO-differentiated cells.

Cell Type	Additives	Total intracellular purines (Adenine+Guanine+Hypoxanthine) pmol/10^6 cells/40 mins		Adenine + Guanine pmol/10^6 cells/40 mins	
		Exp 1	Exp 2	Exp 1	Exp 2
Undifferentiated HL60 cells	None	1418	1340	205	183
Undifferentiated HL60 cells	Phenazine methosulphate (0.1mM)	951	1123	261	305
DMSO-differentiated cells	None	768	775	220	178
DMSO-differentiated cells	Phenazine methosulphate (0.1mM)	977	1252	214	215
PMNs	None	674	842	40	30

Cells were incubated in the presence of [^{14}C]-hypoxanthine (300 nmole, 51μCi/mmole), glucose (10mM), glutamine (3mM) and dialyzed foetal calf serum (10%) and the synthesis of purines were evaluated as described in the Materials and Methods section. Data are from two separate experiments, each performed in duplicate.

Table 6.6: Labelling of intracellular purines with [^{14}C]-formate by PMNs.

Purine bases	Soluble fraction (1)	RNA (2)	DNA (3)	Sum (1+2+3)	Whole cell	Medium
Adenine	22.65	2.96	0.61	26.22	21.74	14.45
Guanine	2.12	1.12	0.89	4.14	1.82	0.405
Hypoxanthine	0.00	6.15	0.54	6.69	11.8	0.123
All purines	24.77	10.23	2.06	37.05	35.35	14.97

PMNs (10^6 cells/ml) in the presence of 10mM glucose, 3mM glutamine and 10% dialyzed foetal calf serum were labelled with [^{14}C]-formate (320 nmole, 51μCi/μmole). After 40 minutes, two 2-ml aliquots were removed for analysis. The cells in one aliquot were fractionated to determine separately isotopic labelling of purine bases in soluble, RNA and DNA nucleotides. The cells in the second aliquot were hydrolyzed without prior fractionation for determination of labelling of total intracellular purines directly. All values are expressed as picomole of bases/10^6 cells /40 mins and each is the mean of two incubations performed in duplicate. The variation between the data from two experiments was in the range of 5-10%.

compatible with the maximum activities of the salvage pathway enzymes (three to eight-fold lower than in both HL60 cells) (Tables 6 and 6.5).

6.2.6 *De novo* purine and pyrimidine nucleotide synthesis in HL60 and DMSO-treated cells

Both HL60 and DMSO-treated cells incorporated [^{14}C]-$NaHCO_3$ into pyrimidine and purine nucleotides at different rates. The rate of incorporation of [^{14}C]-$NaHCO_3$ into the pyrimidine fraction of DMSO-treated cells was approximately three-fold lower than the rate observed with the HL60 cells. In a similar manner DMSO-induced cells incorporated $^{14}CO_2$ into purines from [^{14}C]-$NaHCO_3$ at a rate six times lower than the HL60 cells (Table 6.7). These results are in accordance with [^{14}C]-formate incorporation, which was also six-fold higher in the undifferentiated HL60 cells.

6.3 Discussion

Cells of the human leukaemic HL60 line undergo morphological maturation and acquire some of the differentiated functions of neutrophils when grown in the presence of DMSO (Chapters 2 and 3). In the present work, levels of purine and pyrimidine nucleotides present in these cells before and after differentiation with DMSO were measured and the activities of the biosynthetic enzymes for purine nucleotide production were estimated. Despite the substantially higher activity of the oxidative segment of the pentose phosphate pathway in DMSO-differentiated cells, the steady-state concentration of ribose 5-phosphate remained significantly lower (Chapter 5). The synthesis of PRPP is dependent on the concentrations of ribose 5-phosphate and ATP and the activity of PRPP synthetase. Its steady-state concentration also depends on the rate of utilization of PRPP; faster utilization of PRPP activates PRPP synthetase whilst its accumulation has the reverse effect and stabilizes the PRPP concentration (Henderson, 1973). In undifferentiated HL60 cells the maximum catalytic capacity of PRPP synthetase was three-fold higher and the ribose-5-phosphate concentration two-fold higher (Chapter 5) than in differentiated cells, yet the steady-state concentration of PRPP remained unchanged (Tables 6 and 6.1). This suggests that PRPP is utilized at a higher rate in the

Table 6.7: [^{14}C]-$NaHCO_3$ incorporation into the pyrimidine and purine fractions of HL60 and DMSO-differentiated cells.

Cell Type	[^{14}C]-$NaHCO_3$ incorporation into the pyrimidine fraction of cells (dpm/10^6cells/60 mins)	[^{14}C]-$NaHCO_3$ incorporation into the purine fraction of cells (dpm/10^6 cells/60 mins)
HL60	1012±88	792±163
DMSO-differentiated cells	371±38*	119±11*

The assay for the labelling of [^{14}C]-$NaHCO_3$ into the pyrimidine and purine fractions of the cells were performed as described in the Materials and Methods section. The values given are the mean ± S.E.M. of six different readings from two separate experiments, each performed in triplicate. *$p<0.01$, significantly different from the corresponding value of control HL60 cells.

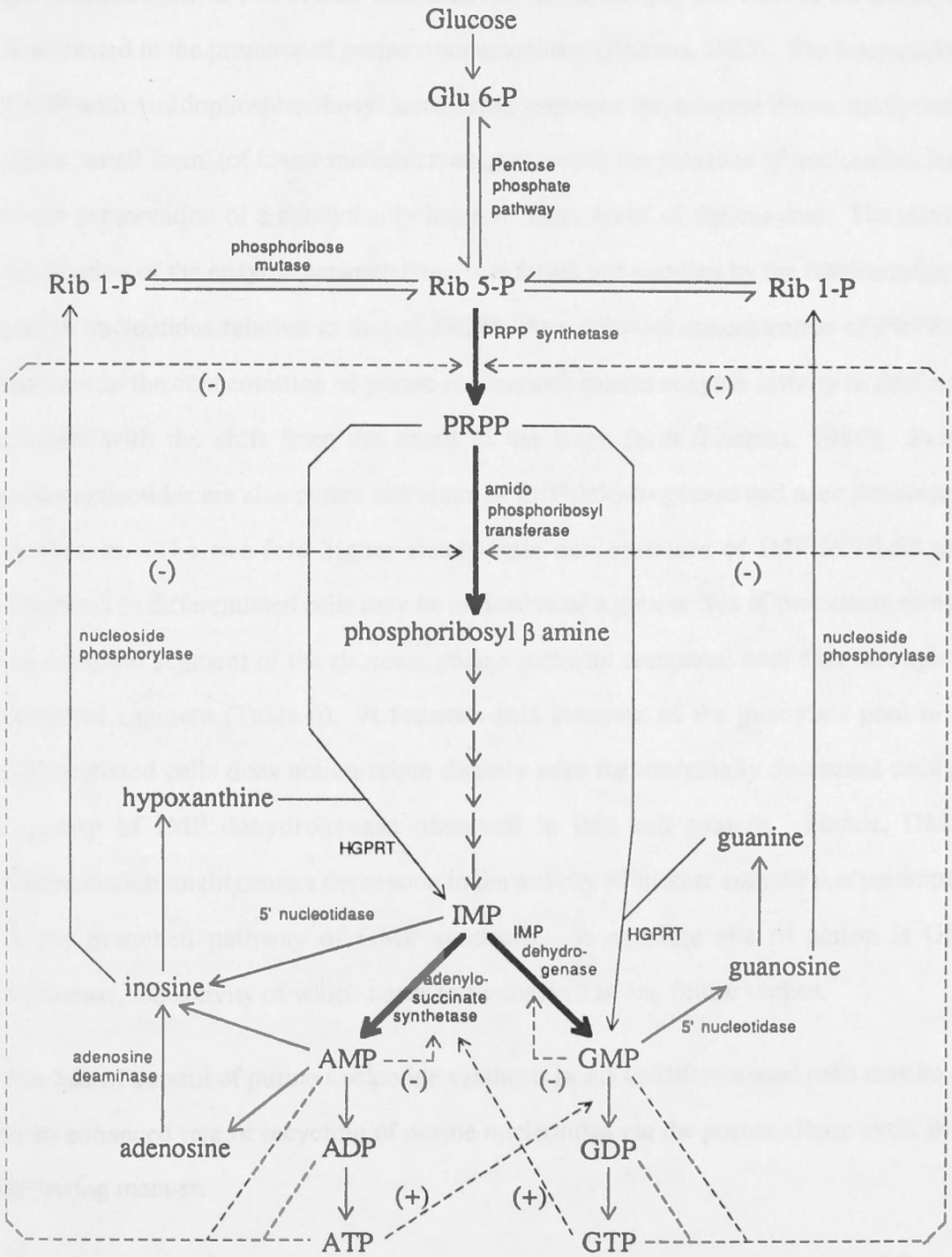
undifferentiated cells, which is borne out and supported by the six-fold and three-fold increases in the incorporation of [^{14}C]-formate and [^{14}C]-bicarbonate into purine and pyrimidine nucleotides (indicative of *de novo* purine and pyrimidine nucleotide synthesis) observed in these cells (Tables 6.2 and 6.7). The distribution of label in the different purine-containing pools showed that the intracellular ATP pool was larger than that of GTP in HL60 cells, with most of the newly-synthesized purine nucleotides being present in the soluble pools (90% in the case of undifferentiated cells and 44% for differentiated cells) which they must traverse before entering the polynucleotides (Tables 6.3 and 6.4). In the case of the undifferentiated cells, only 7-8% of the total purine nucleotides synthesized *de novo* were actually retained in the ribonucleic acid and deoxyribonucleic acid pools, indicating that these cells are producing purine metabolites at a rate grossly in excess of their requirement for incorporation into nucleic acids, a phenomenon commonly observed in other malignant cells (Prajda *et al.*, 1975). A higher rate of incorporation of radioactivity into adenine nucleotides compared with guanine nucleotides does not necessarily implicate IMP dehydrogenase as a rate-limiting step but may occur because of dephosphorylation of xanthosine monophosphate, as is the case with Ehrlich ascites tumour cells which accumulate xanthosine when incubated with [^{14}C]-hypoxanthine (Crabtree and Henderson, 1971). Unaltered labelling of the RNA fraction by [^{14}C]-formate and a three-fold decrease in the incorporation of its radioactivity into the DNA fraction of the differentiated cells (Tables 6.3 and 6.4) possibly indicates that DMSO treatment blocks progression of the cell cycle in the G_1 or early S phase. This is consistent with the inhibition of DNA synthesis observed as [^{3}H]-thymidine incorporation in 9 day DMSO-treated cells (Chapter 2).

The activities of purine nucleotide biosynthetic pathway enzymes became depressed during differentiation by DMSO to levels only slightly higher than those found in normal PMNs (Table 6). The enzymes of the common segment (the sequence of ten reactions that catalyze the synthesis of IMP) and the branched segment (leading to the synthesis of AMP and GMP from IMP) are each under feedback regulation by purine nucleotides (Fig 6.1). Purine nucleoside monophosphates are more potent inhibitors of

Fig 6.1: Purine metabolism in HL60 cells.

Dark arrows indicate the enzymic reactions that are enhanced in HL60 cells. Dotted lines with a (-) sign indicate feed-back inhibition which occurs in normal cells but is absent in HL60 cells. Dotted lines with a (+) sign indicate stimulation by the respective nucleotides.

Fig 6.1


Glucose
Glu 6-P
Pentose phosphate pathway
phosphoribose mutase
Rib 1-P
Rib 5-P
Rib 1-P
PRPP synthetase
(-)
(-)
PRPP
amido phosphoribosyl transferase
(-)
(-)
nucleoside phosphorylase
phosphoribosyl β amine
nucleoside phosphorylase
hypoxanthine
HGPRT
guanine
5' nucleotidase
IMP
IMP dehydro-genase
HGPRT
guanosine
inosine
adenylo-succinate synthetase
5' nucleotidase
adenosine deaminase
AMP
GMP
(-)
(-)
adenosine
ADP
GDP
(+)
(+)
ATP
GTP

amidophosphoribosyl transferase than purine nucleoside diphosphates and triphosphates (Holmes, 1980). Nucleotide inhibition of this enzyme can be overcome by increasing the concentration of PRPP, and cooperativity in the binding of PRPP to the enzyme is accentuated in the presence of purine ribonucleotides (Holmes, 1980). The interaction of PRPP with amidophosphoribosyl transferase converts the enzyme into a catalytically active 'small form' (of lower molecular weight), while the presence of nucleotides leads to the preservation of a catalytically inactive 'large form' of the enzyme. The relative distribution of the enzyme between these two forms is controlled by the concentration of purine nucleotides relative to that of PRPP. At a constant concentration of PRPP, an increase in the concentration of purine nucleotides causes enzyme activity to decline in parallel with the shift from the small to the large form (Holmes, 1980). Purine mononucleotides are also potent inhibitors of IMP dehydrogenase and adenylosuccinate synthetase. The two-fold higher steady-state concentration of IMP in HL60 cells compared to differentiated cells may be indicative of a greater flux of precursors through the common segment of the *de novo* purine pathway compared with flux through the branched segment (Table 6). A fourteen-fold decrease of the guanylate pool in the differentiated cells does not correlate directly with the marginally decreased catalytic capacity of IMP dehydrogenase observed in this cell system. Hence, DMSO differentiation might cause a depression in the activity of another enzyme that participates in the branched pathway of GMP synthesis. A possible site of action is GMP synthetase, the activity of which needs to be checked in any future studies.

The loss of control of purine nucleotide synthesis in the undifferentiated cells may be due to an enhanced rate of recycling of purine nucleotides via the purine-ribose cycle in the following manner:

Purine nucleotide → Purine nucleoside → Ribose 1-phosphate → Ribose 5-phosphate → PRPP → Purine nucleotide.

Continuous operation of this cycle would enable the undifferentiated cells to maintain a sufficiently low steady-state concentration of purine nucleotides to avoid feedback

inhibition of PRPP synthetase and/or amidophosphoribosyl transferase. The increased rate of PRPP synthesis resulting from enhanced ribose 5-phosphate concentration would also activate amidophosphoribosyl transferase and increase the rate of purine biosynthesis *de novo* in HL60 cells. This assumption is consistent with the release to the incubation medium of 44% of the total purine nucleotides synthesized by the undifferentiated cells (Table 6.3). In the differentiated cells, although the purine-ribose cycle is potentially operative (as evidenced by the presence of the enzymes), the concentration of ribose 5-phosphate is not raised to the same extent as that found in the undifferentiated cells. This may be due to the lower overall rate of purine nucleotide synthesis *de novo*, of which 38% is released into the incubation medium .

Studies with [^{14}C]-hypoxanthine showed that a very active salvage pathway, which remained unaltered after differentiation, also exists in HL60 cells (Table 6.5). This observation is supported by the activities of the salvage enzymes HGPRT and APRT, which were unaffected by differentiation. In differentiated cells, this pathway is the major route by which purine nucleotides are synthesized. It has the capacity to contribute 90% of the total purine nucleotides synthesized compared to 58% in the undifferentiated cells (Table 6.2 and 6.5). Similar observations have been reported for mitogen stimulated rat thymocytes where the *de novo* purine and pyrimidine nucleotide synthesis are markedly lower than those of the salvage pathway(Schöbitz *et al.*, 1991). The greater synthesis of purine nucleotides by the salvage pathway compared to the *de novo* pathway may be ascribed to a higher affinity of the salvage enzymes for the shared substrate PRPP than that possessed by amidophosphoribosyl transferase. In the case of hepatomas, HGPRT had approximately 100-fold higher affinity than amidophosphoribosyl transferase for PRPP (Weber *et al.*, 1983 and 1977). In HL60 and DMSO-treated cells the activity of HPGRT is two to three-fold higher than that of amidophosphoribosyl transferase, which may explain why the synthesis of a greater proportion of the purine nucleotides occurs by the salvage pathway. Even though hypoxanthine was taken up at double the rate by the undifferentiated cells, the total synthesis of adenine and guanine nucleotides by both cell types remained the same.

Enhanced uptake of purine bases and nucleosides compared to that measured in quiescent erythrocytes and granulocytes has also been observed in proliferating leukaemic cells (K562 and HL60 cells) (Muller *et al.*, 1983). This might be due to a gradual decrease in the number of specific binding sites per cell for hypoxanthine transport as the cell volume decreases with differentiation (Chen *et al.*, 1986; Ahmed *et al.*, 1991). The intracellular accumulation of hypoxanthine by the undifferentiated cells suggests that the rate of transport of hypoxanthine may exceed the maximum velocity of intracellular phosphoribosylation. Purine bases cross the cell membrane by facilitated diffusion and are normally trapped in the cytoplasm immediately after interaction with phosphoribosyl transferases and intracellular substrates. Comparison of the kinetics of purine transport and intracellular phosphoribosylation have shown that the activities of the intracellular enzymes are rate-limiting for the uptake process. Since phosphate stimulates the uptake of purine bases, the limited availability of PRPP has been suggested to play a regulatory role in intracellular phosphoribosylation (Muller *et al.*, 1983). The activities of phosphoribosyl transferases in cell lysates were found to be two-fold to fifty-fold higher than their apparent activities *in situ*, indicating that, under physiological conditions, phosphoribosyl transferases are not saturated with respect to PRPP (Muller *et al.*, 1983).

In this study the effect of phenazine methosulphate on the synthesis of PRPP and purine nucleotides was measured both before and after differentiation (Tables 6.1, 6.2 and 6.5). Phenazine methosulphate activated the oxidative pentose phosphate pathway in both undifferentiated and differentiated cells by acting as an electron acceptor for NADPH, thus increasing the availability of ribose-5 phosphate within the cytoplasm. The stronger effect of phenazine methosulphate on the oxidative pentose phosphate pathway in the undifferentiated cells is indicative that they have reserve enzymic capacity which allows their pathway to reach the same level as that of the differentiated cells (Fig 6). The activation of the oxidative pentose phosphate pathway by phenazine methosulphate resulted in an increased steady-state concentration of PRPP, which increased approximately four to five-fold in both cell types (Table 6). These findings suggest that the oxidative segment of the pentose phosphate pathway may be a major route of PRPP

synthesis in HL60 cells and that the concentration of ribose 5-phosphate is not normally saturating for the synthesis of PRPP. Increased synthesis of PRPP in the presence of phenazine methosulphate led to increased accumulation of extracellular purines while the intracellular concentration remained constant (Table 6.2) so that the overall effect was one of stimulation. The stimulatory effect on the rate of synthesis of extruded purines may be due to restricted rephosphorylation of purine mononucleotides (AMP and GMP), leading to their net breakdown by the purine-ribose cycle and their subsequent release into the medium as purine bases. This sort of energy dissipative consequence of phenazine methosulphate has also been observed when 3T6 fibroblasts are treated with methylene blue (Buchanan *et al.*, 1982).

The presence of glutamine increased the steady-state concentration of PRPP in the HL60 cells. In the case of the undifferentiated cells, this may have been due to a differentially higher flux of glucose through the oxidative pentose phosphate pathway if inhibition of glycolysis occurred at the phosphofructokinase locus (Chapter 5). This interpretation is consistent with the two-fold increase in the C_1/C_6 ratio (Fig 6) when glutamine is present. In the absence of exogenous glutamine, the *de novo* pathway of purine nucleotide synthesis was restricted to 30% of its maximum activity in the undifferentiated cells – not only because the three glutamine-requiring enzymes were deprived of adequate substrate in its absence – but also because of the decreased availability of PRPP due to the putative release of inhibition of glycolysis at the phosphofructokinase locus (Chapter 5, Ahmed *et al.*, 1993) (Table 6.1).

In PMNs separated from human blood the activity of the *de novo* pathway was three-fold lower than that in the undifferentiated cells but twice as high as that in the DMSO-differentiated cells. The activities of PRPP synthetase and amidophosphoribosyl-transferase were five to twelve-fold and two to three-fold lower in PMNs than in the undifferentiated and differentiated HL60 cells respectively. On the other hand, PMNs synthesized purine nucleotides by the salvage pathway at only 20% of the rate of HL60 cells and the activities of the enzymes HGPRT and APRT were three to eight-fold lower. Unlike DMSO-differentiated cells, the contributions of the *de novo* and salvage pathways

to the synthesis of purine nucleotides in freshly-isolated PMNs were approximately equal.

Alteration of purine and pyrimidine metabolism in differentiating HL60 cells provides evidence that stimulation by DMSO results in the switching on of some of the regulatory mechanisms which normally limit the rate of *de novo* purine nucleotide synthesis in circulating PMNs. The regulatory mechanisms that control the expression of the activities of *de novo* pathway enzymes are altered markedly on differentiation, yet the expression of the genes which control the activities of the salvage pathway enzymes remain unaltered (Reem and Friend, 1975). DMSO-differentiated cells may behave like PMNs phenotypically (Ahmed *et al.*, 1991) but, as shown here, they retain striking metabolic differences that reflect their distinctive origin. These findings suggest that factors which regulate gene expression during normal development can, when activated in leukaemic cells, partially modify the expression of the malignant phenotype without fully expressing the characteristics of the normal phenotype. HL60 cells in culture may provide a useful model for studying gene expression under conditions that can be subjected to considerable experimental control.

Chapter 7: Effect of different chemotherapeutic agents on differentiation and purine/pyrimidine metabolism in HL60 cells

7 Introduction

Leukaemia is caused by mutations in genes that control the growth and division of cells (Section 1.5.1). Since leukaemic cells arise from normal cells when mutational changes are induced by carcinogens (which might be chemicals or viruses) there are likely to be immunological and metabolic differences which can be exploited therapeutically. The division of rapidly-proliferating leukaemic cells can only occur after the duplication of the cell's chromosomes. Nucleotides synthesized as ribonucleotides via the purine and pyrimidine biosynthetic pathways can be incorporated into RNA or converted to deoxyribonucleotides for utilization in DNA synthesis. Blockage by a specific inhibitor of a particular enzyme-catalyzed reaction of one of these pathways would result in accumulation of intermediates proximal to the blockage and depletion of downstream intermediates *en route* to the synthesis of nucleic acids. As a result, a 'metabolic cross-over point' is likely to be observed at the site of inhibition when the concentrations of sequential pathway intermediates in untreated leukaemic cells are compared with those of cells treated with the inhibitors (Lyons *et al.*, 1990). In some cases the inhibitors are not effective against growing leukaemic cells because of 'metabolic resistance' (Christopherson and Duggleby, 1983), a phenomenon which might occur if the normal intermediate accumulates to concentration sufficient to overcome the blockage by competing directly with the inhibitor of the target enzyme.

Inhibitor-induced deficiency of nucleotides may cause normal cells to enter the quiescent G_0 phase of the cell division cycle. In contrast, leukaemic cells – having lost the ability to enter the G_0 phase when conditions for growth become unfavourable (Christopherson and Lyons, 1990) – continue to cycle until they exhaust their nucleotide pool and die. The pathways of nucleotide biosynthesis are active during the S phase of the cell cycle. Only cells in S phase are susceptible to inhibitors of these pathways; normal cells resting in the G_0 phase are not. As a result, cells exposed to the inhibitors will be arrested in the S phase of the cell cycle and will subsequently die. A number of potent inhibitors of nucleotide biosynthesis have been discovered; some are in clinical use as antileukaemic

drugs, or are undergoing clinical trials, while others have only been used in experimental systems *in vitro* (e.g., with cultured leukaemic cells). Such inhibitors can be classified into three broad categories:

(1) Natural products;

(2) Antimetabolites, which are chemical analogues of metabolites essential for the growth of leukaemic cells; and

(3) Inhibitors which act at the active site(s) of the target enzymes of nucleotide biosynthetic pathways.

The properties and plausible mechanisms of action of four such inhibitors will be discussed in this chapter.

(1) Mycophenolic acid

Mycophenolic acid was first isolated in 1896 from the culture filtrate of *Penicillium stoloniferum*, and its structure (Fig 7 a) was determined by Birkinshaw and his colleagues in 1952 (Birkinshaw *et al.*, 1952). This antibiotic interferes with the interconversion of inosine, xanthosine and guanosine monophosphates. IMP dehydrogenase (which converts IMP to XMP) and GMP synthetase (which converts XMP to GMP) are inhibited by mycophenolic acid by the mechanism of 'mixed inhibition' (Franklin and Cook, 1969; Sweeney *et al.*, 1972). This antibiotic inhibits DNA synthesis and thus exhibits antitumour activity against a range of experimental tumours in animals (Sweeney *et al.*, 1969; Williams *et al.*, 1968). The inhibition of cell growth is reversed by guanine but not by hypoxanthine, xanthine or adenine. When used to treat experimental animals, mycophenolic acid is secreted into the bile and excreted in the urine and faeces as glucuronide (Sweeney *et al.*, 1972). Some murine leukaemia and ascites cells are resistant to mycophenolic acid; they usually have a high activity of the enzyme HGPRT which facilitates the 'salvaging' of guanine to form GMP and circumvents the inhibition of IMP dehydrogenase and GMP synthetase. Other resistant

tumour cells have a low activity of β-glucuronidase. This results in a low rate of hydrolysis of mycophenolic acid-glucuronide and thereby favours the maintenance of a low intracellular concentration of free mycophenolic acid (Sweeney *et al.*, 1972).

(2) Alanosine

Alanosine was first isolated in 1966 from cultures of *Streptomyces alanosinicus* (Murthy *et al.*, 1966). The structure of this antibiotic is shown in Fig 7 b. In Ehrlich ascites cells growing in mice, alanosine was shown to inhibit the incorporation of [^{14}C]-formate and [^{14}C]-glycine into AMP without affecting the incorporation of these precursors into GMP (Gale and Schmidt, 1968). This indicates that the antibiotic, or one of its metabolites, exerts its inhibition at the level of adenylosuccinate synthetase, the penultimate enzyme involved in the synthesis of adenine nucleotides. It was found later that alanosine itself was not the inhibitor of this enzyme, but rather an anabolite of the inhibitor: alanosine in conjugation with AICOR (5 amino-4-imidazole carboxylic acid ribonucleotide, an intermediate in the pathway of purine biosynthesis) forms a compound which functions as the actual antimetabolite (Hurlbert *et al.*, 1977) (Fig 7.1). The enzyme responsible for this specialized reaction is SAICAR synthetase (5-amino-4-imidiazole-N-succinocarboximide ribonucleotide synthetase) and the product, alanosyl-AICOR (alanosyl-5-amino-4-imidiazole carboxylic acid ribonucleotide) is a powerful inhibitor *in vivo* and *in vitro* of adenylosuccinate synthetase. A partially-purified preparation of adenylosuccinate synthetase from leukaemic L5178Y cells has been used to show that alanosyl-AICOR is a non-competitive inhibitor with respect to aspartic acid and GTP as variable substrates, but a competitive inhibitor with IMP (Tyagi and Cooney, 1983 and 1984). Alanosine inhibited the incorporation of [^{14}C]-aspartic acid into RNA of *Candida albicans*, and aspartic acid antagonised the inhibition of growth by alanosine in these cells (Gale *et al.*, 1968); treatment of the same cells with alanosine also resulted in the inhibition of aspartate transcarbamoylase, the key enzyme involved in *de novo* pyrimidine synthesis. In Novikoff rat hepatoma cells alanosine inhibited the transport of aspartic acid across the plasma membrane (Graff and Plagemann, 1976). Clinical trials

with alanosine have revealed only minimal anti-tumour activity in solid tumours and leukaemia cells, with some side effects which overshadow its therapeutic value (Ahluwalia *et al.*, 1990); these properties have led to the abandonment of further clinical investigation. However, it still remains an important antimetabolite for unravelling *in vitro* the complexities associated with the uncontrolled perpetuation of nucleic acid biosynthesis in neoplasia.

(3) Acivicin

Acivicin is a cyclic analog of L-glutamine identified in the fermentation broth of *Streptomyces sviceus* (Hanka and Dietz, 1973); its structure is shown in Fig 7 c. Because of its steric similarity to glutamine, acivicin strongly inhibits a number of glutamine-requiring enzymes, especially the rate-limiting enzymes of *de novo* purine nucleotide synthesis (amidophosphoribosyl transferase, formylglycinamidine ribosylphosphate synthetase and GMP synthetase) and pyrimidine nucleotide synthesis (carbamoyl phosphate synthetase and CTP synthetase) (Aoki *et al.*, 1982; Lui *et al.*, 1982; Weber *et al.*, 1982). In acivicin-treated cells, such inhibition correlates with marked decreases in the CTP, GTP, dCTP, dGTP and dTTP pools, with a concomitant elevation of the UTP pool (Lyons *et al.*, 1990). Acivicin is not only a competitive inhibitor of glutamine-requiring enzymes but it also inactivates these target proteins directly by binding reversibly to, and initiating alkylation of, the glutamine-binding site, probably at a cysteinyl residue (Tso *et al.*, 1980; Allen *et al.*, 1981). *In vivo*, acivicin causes rapid inactivation of amidophosphoribosyl transferase of hepatoma 3924A, whereas the activity of the host (rat) liver enzyme is affected only minimally (Prajda, 1985). The greater sensitivity of the neoplastic cells compared with normal liver has been attributed to a ten-fold higher content of glutamine in the latter (0.5mM in the hepatoma versus 5mM in liver). Preclinical trials with acivicin in animal models have revealed a broad spectrum of antitumour activity. The drug is effective, for example, against leukaemias and solid tumours implanted in nude mice (Houchens *et al.*, 1979). However clinical trials in humans have shown minimal to no activity against numerous neoplasms

Fig 7: Chemical structures of inhibitors:

(a) mycophenolic acid (6-[4-hydroxy-6-methoxy-7-methyl-3-oxo-5-phthalanyl]-4-methyl-4-hexenoic acid; NSC 129,185); **(b)** alanosine (L-2 amino-3-[N-hydroxy-N-nitrosamino] propionic acid; NSC 153,353); **(c)** acivicin (L-[aS, 5S]-a-amino-3-chloro-4,5-dihydro-5-isoxazolacetic acid; NSC 163,501); and **(d)** dipyridamole (2,6-bis-diethanolamino-4,8-dipiperidinopyrimido-[5,4-d]-pyrimidine).

Fig 7 (a)

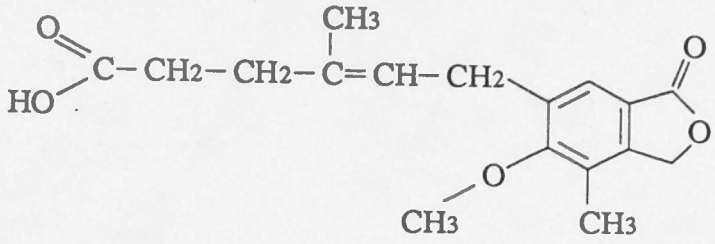


Fig 7 (b)

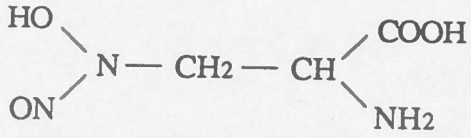


Fig 7 (c)

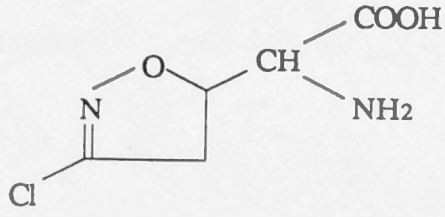


Fig 7 (d)

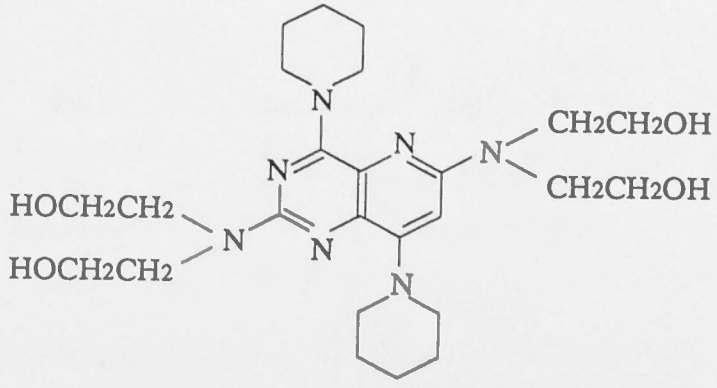
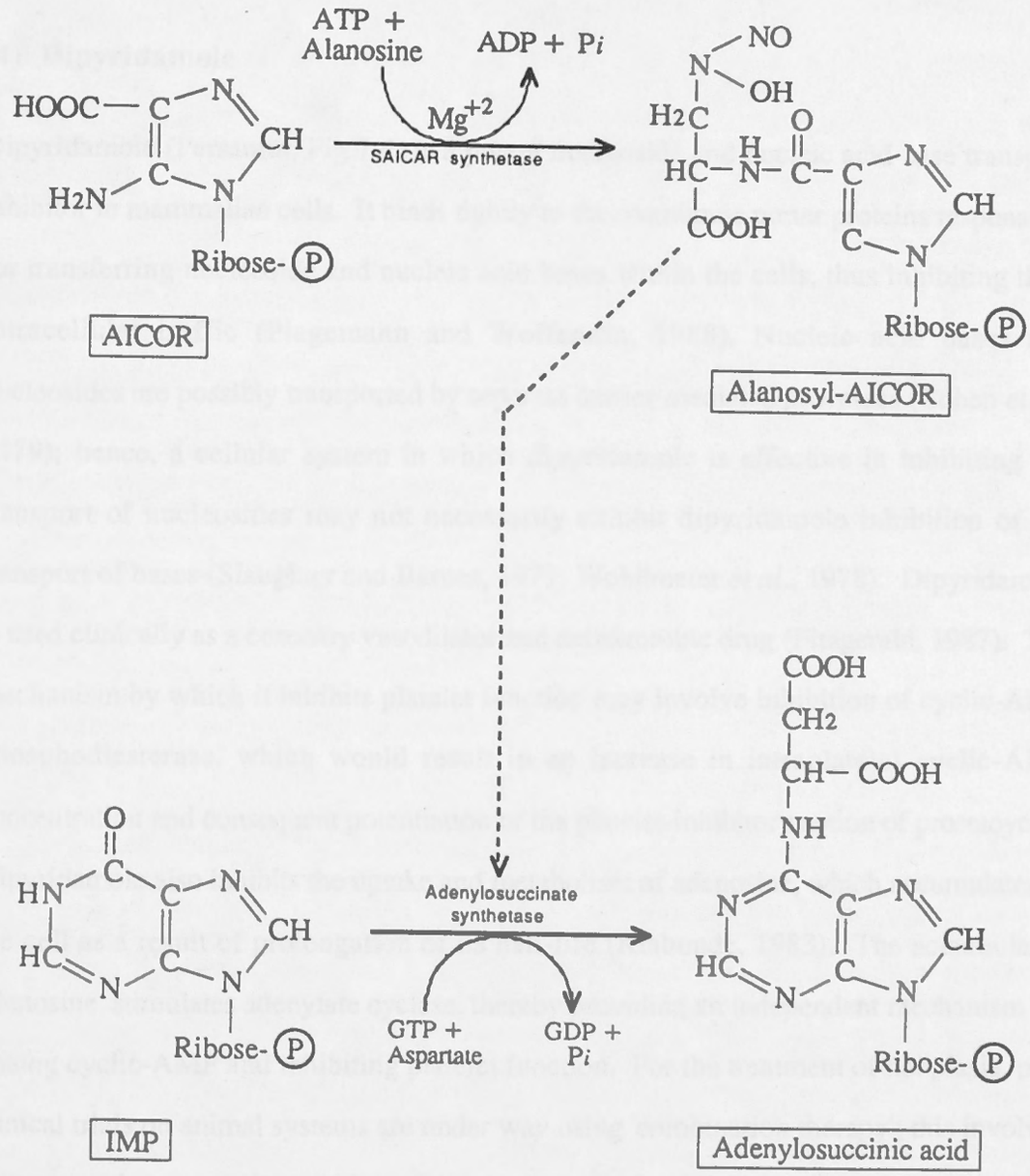


Fig 7.1: Mechanism of action of alanosine.

Alanosine combines with AICOR (5 amino-4-imidazole carboxylic acid ribonucleotide) to form alanosyl-AICOR (alanosyl-5-amino-4-imidiazole carboxylic acid ribonucleotide) which is a powerful inhibitor of adenylosuccinate synthetase. The reaction is catalyzed by SAICAR synthetase (5-amino-4-imidiazole-N-succinocarboximide ribonucleotide synthetase). Dotted line indicates the inhibitory action imposed by alanosyl-AICOR on adenylosuccinate synthetase.

Fig 7.1


ATP +
Alanosine
ADP + Pi
Mg+2
SAICAR synthetase
HOOC
H2N
Ribose-P
AICOR
NO
OH
H2C
HC
COOH
Ribose-P
Alanosyl-AICOR
Adenylosuccinate
synthetase
GTP +
Aspartate
GDP +
Pi
Ribose-P
IMP
COOH
CH2
CH— COOH
NH
HC
Ribose-P
Adenylosuccinic acid

(Ahluwalia *et al.*, 1990). The inhibitor is now being tested in combination with several other agents; the nucleoside transport inhibitor dipyridamole is one of them (Fischer *et al.*, 1986).

(4) Dipyridamole

Dipyridamole (Persantin, Fig 7 d) is a potent nucleoside and nucleic acid base transport inhibitor in mammalian cells. It binds tightly to the membrane porter proteins responsible for transferring nucleoside and nucleic acid bases within the cells, thus inhibiting their intracellular traffic (Plagemann and Woffendin, 1988). Nucleic acid bases and nucleosides are possibly transported by separate carrier-mediated processes (Cohen *et al.*, 1979); hence, a cellular system in which dipyridamole is effective in inhibiting the transport of nucleosides may not necessarily exhibit dipyridamole inhibition of the transport of bases (Slaughter and Barnes, 1979; Wohlhueter *et al.*, 1978). Dipyridamole is used clinically as a coronary vasodilator and antithrombic drug (Fitzgerald, 1987). The mechanism by which it inhibits platelet function may involve inhibition of cyclic-AMP phosphodiesterase, which would result in an increase in intraplatelet cyclic-AMP concentration and consequent potentiation of the platelet-inhibitory action of prostacyclin. Dipyridamole also inhibits the uptake and metabolism of adenosine, which accumulates in the cell as a result of prolongation of its half-life (Klabunde, 1983). The accumulated adenosine stimulates adenylate cyclase, thereby providing an independent mechanism for raising cyclic-AMP and inhibiting platelet function. For the treatment of neoplasia, pre-clinical trials on animal systems are under way using 'combination therapy'; this involves using, together, an antimetabolite of the *de novo* nucleotide biosynthesis pathway, such as acivicin, and an inhibitor of the salvage nucleotide pathway, such as dipyridamole, to provide synergistic cytotoxic action against neoplastic cells. Such a synergistic response – with its predicted effect on the nucleotide pools – has been observed in studies *in vivo* with the transplanted hepatoma 3924A (Weber *et al.*, 1983). These results may have some clinical relevance and such combination therapy awaits further clinical trials.

Aim of the Study

Due to the drastic inhibition of purine and pyrimidine nucleotide biosynthesis in DMSO-differentiated cells (Chapter 6, Ahmed and Weidemann, 1994) and because of the significant role played by purine and pyrimidine analogues in cellular differentiation (Lucas *et al.*, 1983), the effect of four different nucleotide biosynthesis inhibitors were tested on differentiation and purine/pyrimidine metabolism in HL60 cells. This chapter describes the mechanism of action of four potent inhibitors – acivicin, mycophenolic acid, alanosine and dipyridamole – of nucleotide biosynthesis. These inhibitors are known to display pharmacological profiles as antitumour agents (Christopherson and Lyons, 1990). The main purpose of this study was to investigate the biochemical effect of total purine depletion caused by acivicin, with those caused by the specific depletion of guanine nucleotides by mycophenolic acid and of adenine nucleotides caused by alanosine in order to compare the relative impact of adenine and guanine nucleotide on cellular proliferation and differentiation. Hence, experiments were designed to assess the purine and pyrimidine nucleotide biosynthesis using [^{14}C]-formate, [^{14}C]-hypoxanthine and Na$H^{14}CO_3$ (Section 7.1.3). The inhibitory points in the biosynthetic pathways were evaluated by measuring the activities of the key regulatory enzymes in the pathways (Section 7.1.3) and also by measuring the level of important metabolites accumulated at the points of inhibition (Section 7.1.3). The assessment of differentiation or the extent of proliferation of the inhibitor-treated cells was evaluated by morphological examination (Section 7.1.3), [^{14}C]-thymidine and [^{3}H]-uridine incorporation and by measuring the respiratory burst activity – done by luminol-dependent CL and cytochrome C reduction – (Section 7.1.3). In each of these experiments HL60 cells were used as control. These experiments would help to identify the site of inhibition exerted by these inhibitors in purine/pyrimidine nucleotide biosynthetic pathways and thus will help in elucidating the mechanism by which these inhibitors induce differentiation and stop proliferation in HL60 and other leukaemic cells.

7.1 Materials and Methods

7.1.1 Chemicals

Dipyridamole or persantin (2,6-bis-diethanolamino-4,8-dipiperidinopyrimido-[5,4-d]-pyrimidine), acivicin (L-[αS, 5S]-α-amino-3-chloro-4,5-dihydro-5-isoxazolacetic acid; NSC 163,501) and mycophenolic acid (6-[4-hydroxy-6-methoxy-7-methyl-3-oxo-5-phthalanyl]-4-methyl-4-hexenoic acid; NSC 129,185) were obtained from Sigma Chemical Company St Louis, USA. Alanosine (L-2-amino-3-[N-hydroxy-N-nitrosamino] propionic acid; NSC 153,353) was a gift from Dr R.I. Christopherson, Sydney University, Australia. The rest of the chemicals, radioisotopes and enzymes were obtained from the sources described in Section 6.1.1.

7.1.2 Cells

HL60 cells were maintained as described in Section 2.1.2. For determination of the inhibitor concentration at which cell growth was inhibited by 50% (IC_{50}), HL60 cells were inoculated into the growth medium at a concentration of 3-5 x 10^5 cells/ml. Aliquots (0.4 ml) of the inhibitors at different concentrations, dissolved in either absolute ethanol or distilled water (both sterile), were dispensed into 75 cm^2 flasks containing cells, so that the cells were exposed to the appropriate inhibitor concentrations. The cells treated with the inhibitors were incubated at 37^0C in an atmosphere of 5% CO_2 and 95% air for 3-4 days. Inhibitor treated-cells were collected, washed with PBS and the cell densities determined with a haemocytometer using the trypan blue (0.1%) exclusion method. For certain studies, cells were exposed to inhibitors in a growth medium supplemented with inosine or hypoxanthine or adenosine or adenine or guanosine or guanine. The bases and nucleosides were dissolved in sterile distilled water to prepare stock solutions (10mM), which were further diluted in culture medium to achieve the final concentration of 80μM. All of the bases and nucleosides except guanine were readily soluble in distilled water. Guanine (1ml of 10mM solution) required alkalinization with

KOH (10µl of 10mM solution/ml of guanine solution which was also 10mM) to dissolve it.

7.1.3 Assays performed on the inhibitor-treated cells

Morphological changes in the inhibitor-treated cells were assessed by light microscopy using oil and a magnification of x 1000. For this purpose, cytospin slides were prepared and stained with Diff Quick as described in Section 2.1.3.

For assessment of the differentiation of the inhibitor-treated cells, luminol-dependent CL and cytochrome C reduction assays were performed in parallel as described in Sections 2.1.5 and 2.1.6. The inhibition of purine and pyrimidine biosynthesis in the inhibitor-treated cells was assessed by the assays described in Sections 6.1.4, 6.1.5 and 6.1.6. The assays for PRPP and IMP were performed by the methods described in Section 6.1.9. Pentose 5-phosphates were measured by the method of Racker (1974). All enzymic assays except that of GMP synthetase were performed by the methods described in Sections 6.1.7 and 6.1.8. The assay for GMP synthetase was performed by the method of Martin (1972) with a slight modification: xanthosine monophosphate (0.3mM) and ATP (1mM) were added to the enzymic mixture which contained the other ingredients (except PRPP) at the concentrations described by Martin (1972). The rest of the assay procedure followed the steps described by Martin (1972). Methods for the incorporation of [^{14}C]-thymidine (0.2µCi) and [^{3}H]-uridine (0.2µCi) into the control and inhibitor-treated cells were carried out as described in Section 2.1.14.

7.2 Results

7.2.1 Morphological and phenotypic changes induced in the inhibitor-treated cells

In this chapter the effect of different purine and pyrimidine biosynthesis inhibitors have been evaluated on the differentiation and proliferation of HL60 cells. The IC_{50}(inhibitor concentration at which the growth of the cells is inhibited by 50%) for HL60 cells treated

with mycophenolic acid, acivicin, alanosine and dipyridamole for 3-4 days was approximately 5μM, 50μM, 50μM and 10μM respectively. At these concentrations drastic changes were seen in the nuclei of the inhibitor-treated cells; they exhibited a high degree of irregularity and numerous intranuclear vacuoles, both characteristics that were absent from the nuclei of the original HL60 cells (Figs 7.2 a, b, c and d). A marked reduction in proliferation, assessed by the incorporation of [^{14}C]-thymidine, was observed in all of the inhibitor-treated cells (Table 7). The rate of RNA synthesis was reduced significantly in parallel in the inhibitor-treated cells (Table 7). The extent to which the inhibitors induced differentiation in the HL60 cells was ascertained by measuring the ability of the cells to produce reactive oxygen intermediates in response to PMA, as assessed by luminol-dependent CL (measured in the presence of HRP) and cytochrome C reduction. These assays showed that only mycophenolic acid and acivicin were capable of inducing significant differentiation in HL60 cells (Figs 7.3 and 7.4). Even though cells treated with both of these inhibitors produced superoxide anion (measured by cytochrome C reduction) at the same rate as monocytic cells separated from peripheral human blood, only mycophenolic acid-treated cells were able to produce a luminol-dependent CL response as intense as that generated by authentic mononuclear cells (Figs 7.3 and 7.4; Chapter 2; Figs 2.4 and 2.7). The CL response of acivicin-treated cells to PMA was lower by two-fold than that produced by mycophenolic acid-exposed cells. Since the luminol-dependent CL response was performed in the presence of HRP (which compensates for the lack of myeloperoxidase), and since it measures the co-oxidation of luminol with H_2O_2 and HOCl (the end product of myeloperoxidase reaction), it can be argued that acivicin treatment of HL60 cells resulted in a partial loss of, or failure to induce, superoxide dismutase activity. Alanosine and dipyridamole-treated cells had only a slightly enhanced CL response compared to HL60 cells, but they failed to exhibit any elevation in cytochrome C reduction (Figs 7.3 and 7.4). These results suggest that both acivicin and mycophenolic acid induce a degree of differentiation of HL60 cells while alanosine and dipyridamole have no such effect.

Table 7 : [^{14}C]-Thymidine and [^{3}H]-uridine incorporation in untreated and inhibitor-treated HL60 cells.

Treatment of HL60 cells with inhibitors	[^{14}C]-Thymidine incorporation (% inhibition of control cells)	[^{3}H]-Uridine incorporation (% inhibition of control cells)
None	100	100
Mycophenolic acid	70±3	64±6
Acivicin	62±8	54±16
Alanosine	62±2	58±4
Dipyridamole*	48±4	52±8
Dipyridamole#	88±2	66±3

The concentrations used and the duration of inhibitor treatment were as follows: mycophenolic acid (5μM), acivicin (50μM), alanosine (50μM), dipyridamole* (10μM) and dipyridamole # (50μM). The incorporation of [^{14}C]-thymidine and [^{3}H]-uridine was assessed as described in the Materials and Methods section. The values given are the means ± S.E.M. of at least three separate experiments, each performed in triplicate.

Fig 7.2: Light microscopy showing morphological changes induced in HL60 cells by inhibitors.

(a) Mycophenolic acid-treated cells and; (b) Acivicin-treated cells. Magnification used was x 1000.

Fig 7.2 (a)

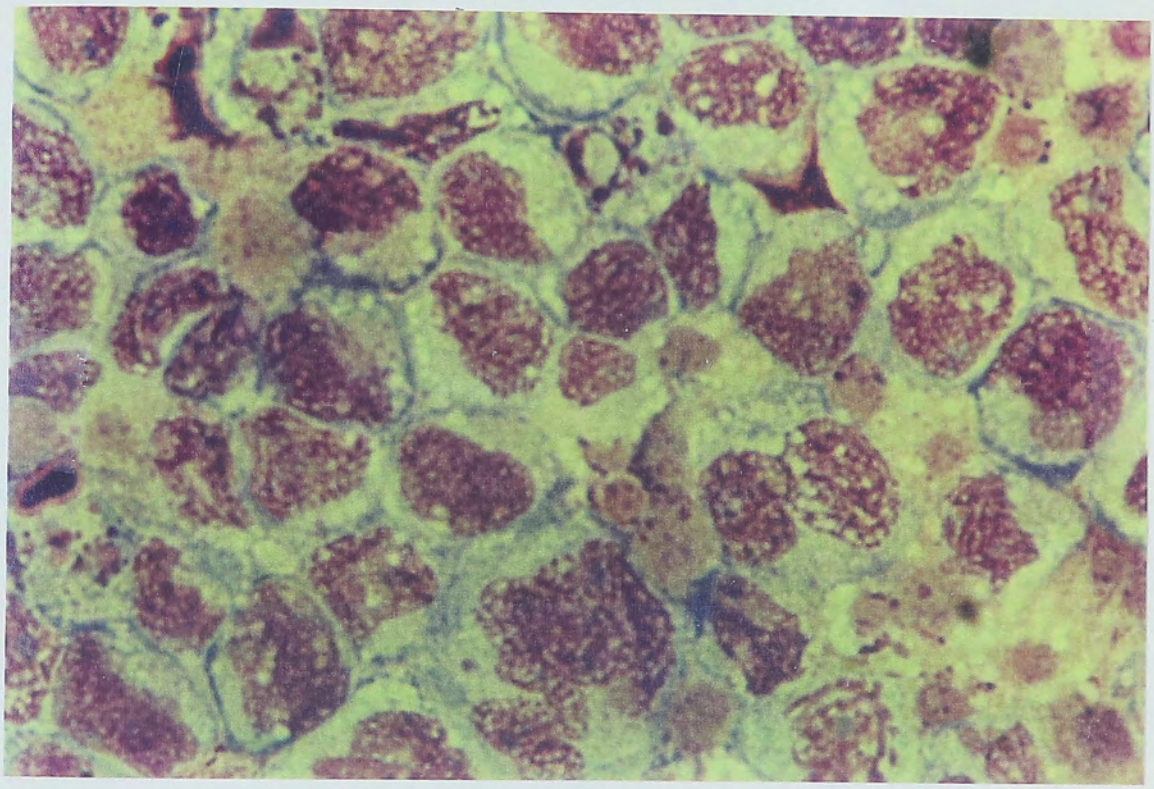


Fig 7.2 (b)

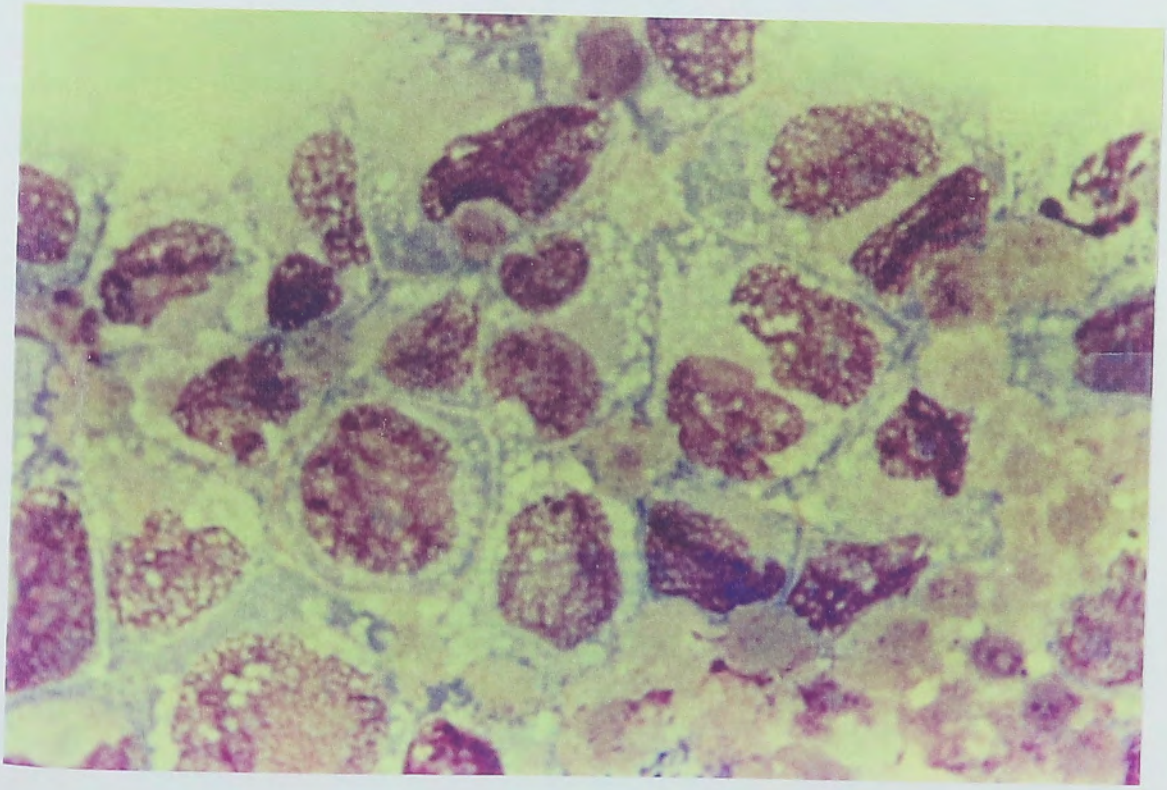


Fig 7.2: Light microscopy showing morphological changes induced in HL60 cells by inhibitors.

(c) Alanosine-treated cells; and **(d)** Dipyridamole-treated cells. Magnification used was x 1000.

Fig 7.2 (c)

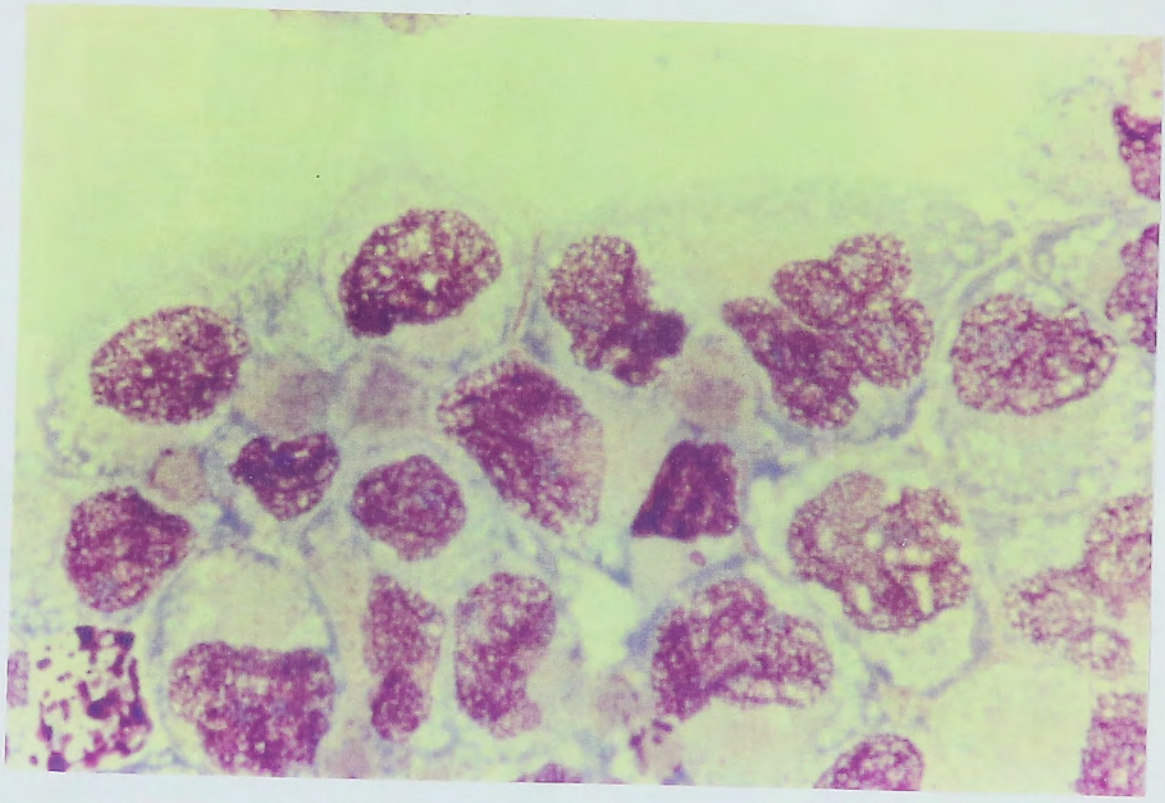


Fig 7.2 (d)

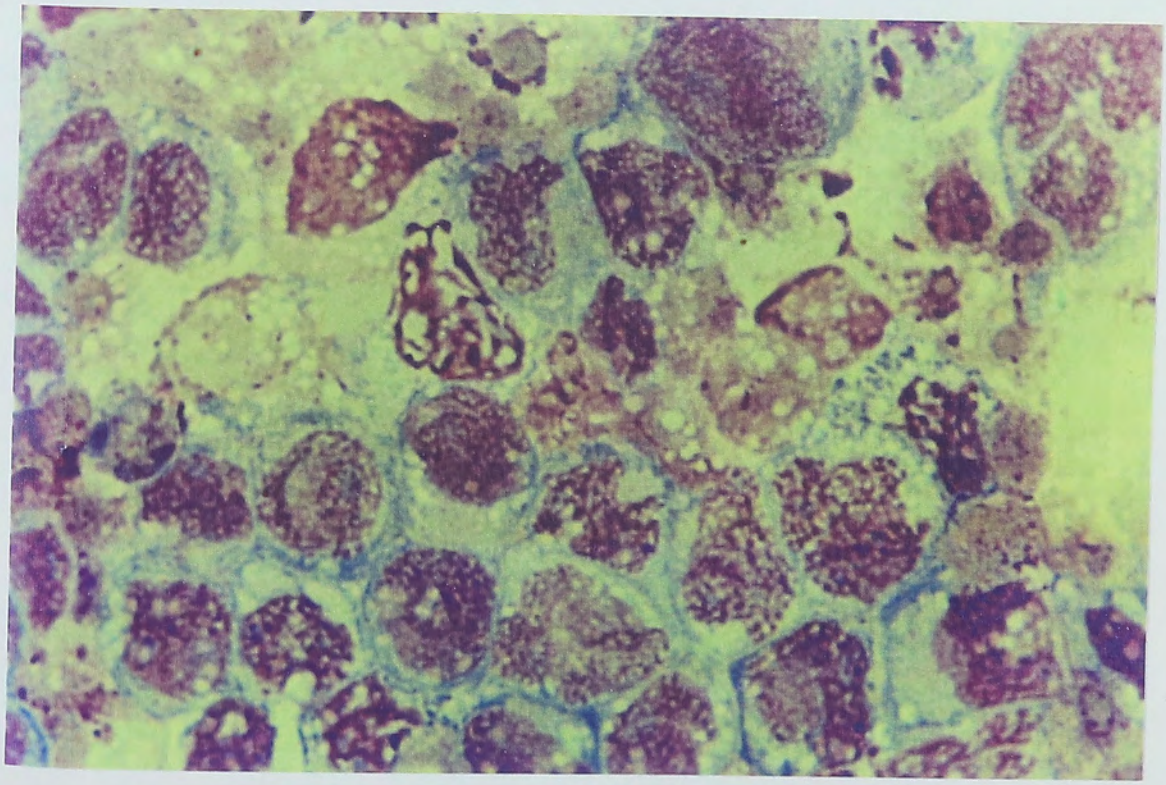


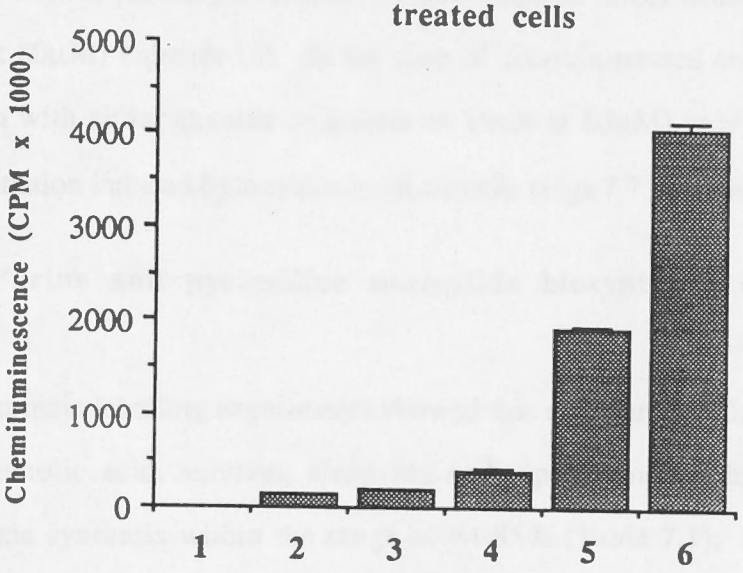
Fig 7.3 : Peak CL response given by HL60 and inhibitor-treated cells.

The conditions for the experiment are described in the Materials and Methods section. The values given are means ± S.E.M. of at least three separate experiments, each performed in triplicate. (1) HL60 cells; (2) dipyridamole (10μM)-treated cells; (3) dipyridamole (50μM)-treated cells; (4) alanosine-treated cells; (5) acivicin-treated cells; and (6) mycophenolic acid-treated cells.

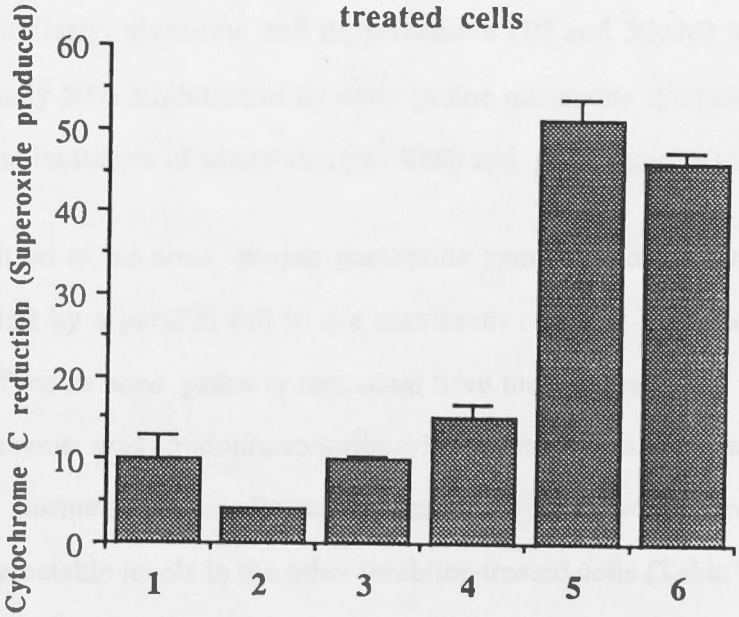
Fig 7.4: Superoxide produced by HL60 and inhibitor-treated cells.

The conditions for the experiment are described in the Materials and Methods section. The values given are means ± S.E.M. of at least three separate experiments, each performed in triplicate. (1) HL60 cells; (2) dipyridamole (10μM)-treated cells; (3) dipyridamole (50μM)-treated cells; (4) alanosine-treated cells; (5) acivicin-treated cells; and (6) mycophenolic acid-treated cells.

(7.3) Peak CL response given by HL60 and inhibitor-treated cells



(7.4) Superoxide produced by HL60 and inhibitor-treated cells



The maturational effect induced by mycophenolic acid could be reversed in the presence of guanine or guanosine (both at 80μM) (Figs 7.5 and 7.6). Hypoxanthine or inosine (both at 80μM) partially overcame the maturational effect while adenine or adenosine (both at 80μM) enhanced it. In the case of acivicin-treated cells, supplementing the medium with either guanine or guanosine (both at 80μM) only partially inhibited the differentiation induced by acivicin in HL60 cells (Figs 7.7 and 7.8).

7.2.2 Purine and pyrimidine nucleotide biosynthesis in inhibitor-treated cells

[^{14}C]-Formate labelling experiments showed that exposure of HL60 cells, separately, to mycophenolic acid, acivicin, alanosine and dipyridamole inhibited *de novo* purine nucleotide synthesis within the range of 44-85% (Table 7.1). Amongst these agents acivicin produced the strongest inhibition of total purine nucleotide biosynthesis *de novo* (85%), of which 90-95% inhibition was seen in the synthesis of both adenylates and guanylates. Mycophenolic acid inhibited *de novo* purine nucleotide synthesis by 44%, with a greater emphasis on the inhibition of guanylates (90%) than that of adenylates (75%). Similarly, alanosine and dipyridamole (10 and 50μM) were responsible for approximately 50% inhibition of *de novo* purine nucleotide synthesis with a fairly equal emphasis on inhibition of adenylates (60-80%) and guanylates (60-90%) (Table 7.1).

The inhibition of *de novo* purine nucleotide synthesis in inhibitor-treated cells was accompanied by a parallel fall in the maximum catalytic activities of key regulatory enzymes of the *de novo* pathway recovered from the cultured cells. The most drastically affected enzyme was amidophosphoribosyl transferase, the first enzyme in the pathway of *de novo* purine synthesis. Its activity fell by 33% in mycophenolic acid-treated cells and to undetectable levels in the other inhibitor-treated cells (Table 7.2). The maximum catalytic activity of PRPP synthetase was less affected, remaining unchanged in mycophenolic acid, alanosine and dipyridamole (50μM)-treated cells and undergoing only small decreases (40%) or increases (32%) following acivicin and dipyridamole (10μM) treatment respectively. Mycophenolic acid and alanosine had an inhibitory effect

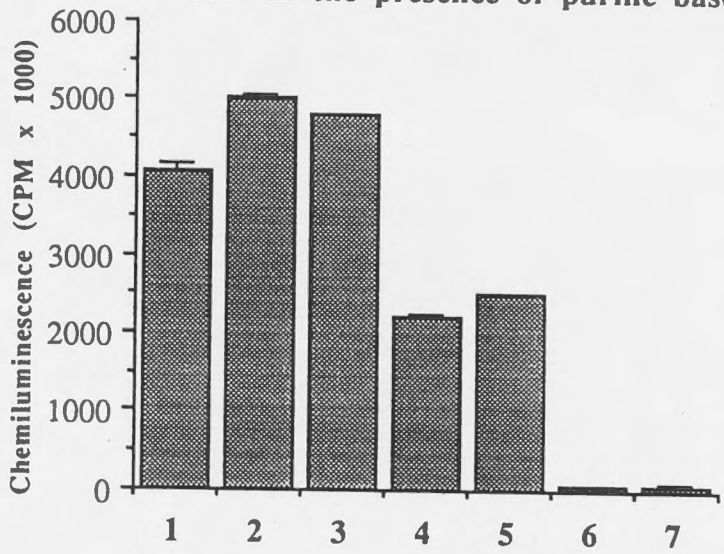
Fig 7.5: Peak CL response given by mycophenolic acid-treated cells in the presence of purine bases and nucleosides.

The conditions for the experiment are described in the Materials and Methods section. The values given are means ± S.E.M. of at least three separate experiments, each performed in triplicate. (**1**) Mycophenolic acid-treated cells; (**2**) [mycophenolic acid + adenosine (80μM)]-treated cells; (**3**) [mycophenolic acid + adenine (80μM)]-treated cells; (**4**) [mycophenolic acid + inosine (80μM)]-treated cells; (**5**) [mycophenolic acid + hypoxanthine (80μM)]-treated cells; (**6**) [mycophenolic acid + guanosine (80μM)]-treated cells; and (**7**) [mycophenolic acid + guanine (80μM)]-treated cells.

Fig 7.6: Superoxide produced by mycophenolic acid-treated cells in the presence of purine bases and nucleosides.

The conditions for the experiment are described in the Materials and Methods section. The values given are means ± S.E.M. of at least three separate experiments, each performed in triplicate. (**1**) Mycophenolic acid-treated cells; (**2**) [mycophenolic acid + adenosine (80μM)]-treated cells; (**3**) [mycophenolic acid + adenine (80μM)]-treated cells; (**4**) [mycophenolic acid + inosine (80μM)]-treated cells; (**5**) [mycophenolic acid + hypoxanthine (80μM)]-treated cells; (**6**) [mycophenolic acid + guanosine (80μM)]-treated cells; and (**7**) [mycophenolic acid + guanine (80mM)]-treated cells.

(7.5) Peak CL response given by mycophenolic acid-treated cells in the presence of purine bases and nucleosides



(7.6) Superoxide produced by mycophenolic acid-treated cells in the presence of purine bases and nucleosides

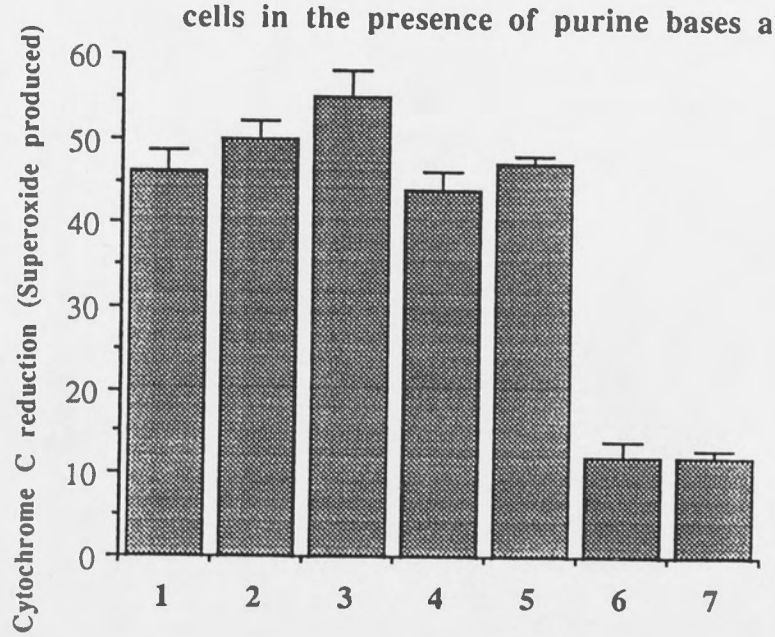


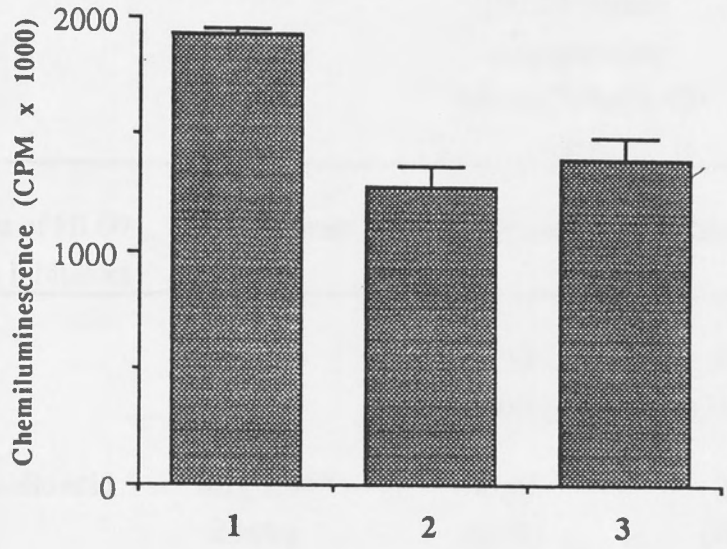
Fig 7.7: Peak CL response given by acivicin-treated cells in the presence of guanine and guanosine.

The conditions for the experiment are described in the Materials and Methods section. The values given are means ± S.E.M. of at least three separate experiments, each performed in triplicate. (1) Acivicin-treated cells; (2) [acivicin + guanosine (80μM)]-treated cells; and (3) [acivicin + guanine (80μM)]-treated cells.

Fig 7.8: Superoxide produced by acivicin-treated cells in the presence of guanine and guanosine.

The conditions for the experiment are described in the Materials and Methods section. The values given are means ± S.E.M. of at least three separate experiments, each performed in triplicate. (1) Acivicin-treated cells; (2) [acivicin + guanosine (80μM)]-treated cells; and (3) [acivicin + guanine (80μM)]-treated cells.

(7.7) Peak CL response given by acivicin-treated cells in the presence of guanine and guanosine



(7.8) Superoxide produced by acivicin-treated cells in the presence of guanine and guanosine

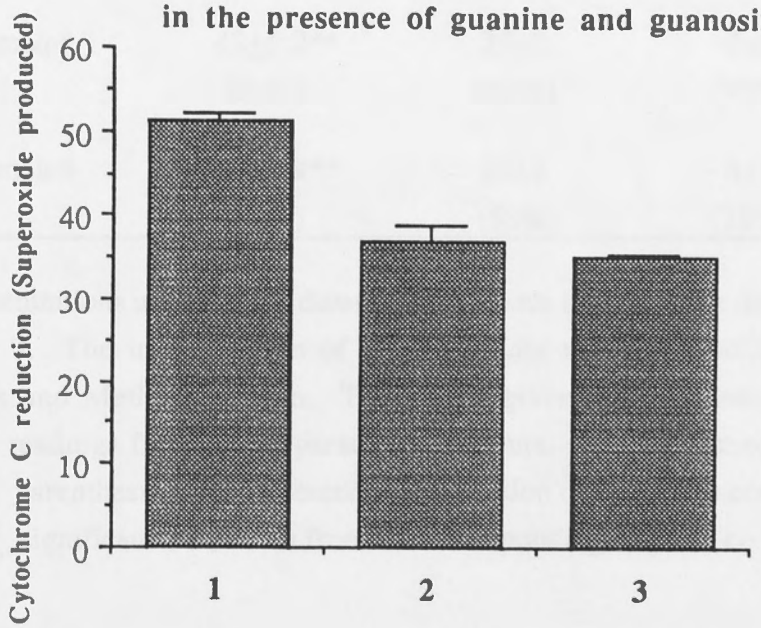


Table 7.1 : [^{14}C]-Formate incorporation in untreated and inhibitor-treated HL60 cells.

Treatment of HL60 cells with inhibitors	[^{14}C]-Formate incorporation (pmole/10^6cells/40 mins) Total purines	Adenylates	Guanylates	Hypoxanthine
None	85±1.2 (100%)	62±2 (100%)	19±1 (100%)	3±1
Mycophenolic acid	48± 2.4** (44%)	15±3 (75%)	2±0 (90%)	30±6
Acivicin	13±0.0** (85%)	3±0 (71%)	9±0 (50%)	8±3
Alanosine	38±4.5** (55%)	11±2 (82%)	7±3 (63%)	21±3
Dipyridamole*	42±5.0** (50%)	25±3 (60%)	2±0 (90%)	18±3
Dipyridamole#	42±0.9** (50%)	26±2 (58%)	4±1 (78%)	10±3

The concentrations used and the duration of inhibitor treatment are described in the legend to Table 7. The incorporation of [^{14}C]-formate was assessed as described in the Materials and Methods section. The values given are the means ± S.E.M. of four different readings from two separate experiments, each performed in duplicate. The values in parenthesis indicate percentage inhibition compared to control untreated cells. **$p<0.01$, significantly different from the corresponding value of control HL60 cells.

Table 7.2 : Maximum catalytic activities of the key regulatory enzymes of purine biosynthesis pathways in untreated and inhibitor-treated cells.

	Enzyme activities ($U/10^6$ cells)						
Treatment of HL60 cells with inhibitors	PRPP synthetase	Amidophospho-ribosyl transferase	IMP dehydrogenase	Adenylosuccinate synthetase	GMP synthetase	HGPRT	APRT
None	0.23±0.01	0.36±0.00	0.25±0.00	0.39±0.017	0.24±0.032	0.43±0.02	0.3±0.00
Mycophenolic acid	0.21±0.01	0.10±0.01**	0.18±0.03	0.32±0.00	0.17±0.00**	1.3±0.00**	0.3±0.00
Acivicin	0.14±0.00	0.00	ND	ND	0.00	0.78±0.12**	0.04±0.00**
Alanosine	0.19±0.01	0.00	0.17±0.01	0.00	ND	0.62±0.01**	0.04±0.00**
Dipyridamole*	0.34±0.00	0.00	0.22±0.02	0.25±0.01	ND	0.72±0.05**	0.07±0.00**
Dipyridamole#	0.17±0.00	0.00	0.24±0.02	0.30±0.02	ND	0.67±0.01**	0.07±0.00**

The concentration and duration of inhibitor treatment are described in the legend of Table 7. The assay procedure for the enzymic activities are described in the Materials and Methods section. Enzymic activities are expressed as $U/10^6$ cells where U=unit of enzyme, expressed as nanomole of substrate formed per minute. Each value is ± S.E.M. of at least three separate experiments, each performed in duplicate. ND=not determined. **$p<0.01$, is significantly different from control HL60 cells.

(approximately 28%) on the maximum catalytic activity of IMP dehydrogenase although the activity of this enzyme remained unchanged upon dipyridamole treatment. GMP synthetase activity was inhibited 25% following mycophenolic acid treatment and even more severely, to virtual undetectability, when the cells were treated with acivicin (Table 7.2). Treatment with alanosine (10μM) for four days caused a 90% fall in the maximum catalytic activity of adenylosuccinate synthetase; the activity became undetectably low when the inhibitor concentration was raised to 50μM for the same length of time. Mycophenolic acid and dipyridamole had no effect on the maximum catalytic activity of adenylosuccinate synthetase.

The activity of HGPRT was enhanced by 67% in mycophenolic acid-treated cells compared with HL60 controls. The total synthesis of purine nucleotides by the salvage pathway fell by 17% in these cells (Table 7.3). The activity of APRT remained unaltered by mycophenolic acid treatment. Acivicin (50μM) and dipyridamole (10μM) had no effect on the uptake of [^{14}C]-hypoxanthine by HL60 cells even though there was a slight enhancement in the synthesis of purine nucleotides by the salvage pathway due to enhanced synthesis (15 to 25%) of adenylates and a concomitant decrease (25 to 50%) in the synthesis of guanylates. Alanosine decreased the synthesis of total purine nucleotides (75%) by the salvage pathway, consistent with the lower uptake of [^{14}C]-hypoxanthine by these cells. On the other hand, treatment of HL60 cells with dipyridamole (50μM) increased the uptake of [^{14}C]-hypoxanthine while the total synthesis of purine nucleotides by the salvage pathway was inhibited. The activity of HGPRT in acivicin, alanosine and dipyridamole-treated cells was enhanced by approximately 30-45%, with a corresponding decrease in the activity of APRT by 75-85% (Table 7.2).

Assessment of the activity of the *de novo* pyrimidine nucleotide pathway by incorporation of [^{14}C]-$NaHCO_3$ showed that the treatment of undifferentiated HL60 cells with the same inhibitors resulted in approximately 30% decrease in *de novo* pyrimidine nucleotide synthesis (Table 7.4). The inhibition of both purine and pyrimidine nucleotide synthesis in the presence of mycophenolic acid, acivicin and alanosine was followed by

Table 7.3 : [^{14}C]-Hypoxanthine incorporation in untreated and inhibitor-treated cells.

		[^{14}C]-Hypoxanthine incorporation (pmole/10^6cell/40 mins)		
Treatment of HL60 cells with inhibitors	[^{14}C]-Hypoxanthine uptake (pmole/10^6 cells/40 mins)	Total purines (adenylates+ guanylates)	Adenylates	Guanylates
None	1386±24	205±7	158±7	47±5
Mycophenolic acid	868±62	191±15	137±12	44±7
Acivicin	1300±50	248±30	213±12	35±8
Alanosine	977±80	155±28	133±18	22±5
Dipyridamole*	1280±90	214±20	190±20	24±8
Dipyridamole#	2005±160	121±18	95±12	26±9

The concentration and duration of inhibitor treatment are described in the legend to Table 7. The incorporation of [^{14}C]-hypoxanthine was assessed as described in the Materials and Methods section. The values given are the mean ± S.E.M. of four different readings from two separate experiments, each performed in duplicate.

Table 7.4 : [^{14}C]-$NaHCO_3$ incorporation into the pyrimidines of untreated and treated cells

Treatment of HL60 cells with inhibitors	[^{14}C]-$NaHCO_3$ incorporation in pyrimidines (dpm/10^6 cells/60 mins)	% of inhibition compared to control cells
None	1012±88	100
Mycophenolic acid	730±30**	28
Acivicin	679±17**	33
Alanosine	768±30**	25
Dipyridamole*	650±28**	35
Dipyridamole#	450±53**	55

The concentration and duration of inhibitor treatment are described in the legend to Table 7. The incorporation of [^{14}C]-$NaHCO_3$ was assessed as described in the Materials and Methods section. The values given are the mean ± S.E.M. of six different readings from two separate experiments, each performed in triplicate. **$p<0.03$, significantly different from the corresponding value of control HL60 cells.

an increase in the steady-state concentration of PRPP (by 1.5 to 3-fold) but no change in the concentration of IMP (Table 7.5). However, a three to seven-fold accumulation of hypoxanthine was observed in the inhibitor-treated cells following labelling with [^{14}C]-formate (Table 7.1). No PRPP could be detected in cells treated with 10μM dipyridamole and only about one third of the PRPP level present in control HL60 cells was found when the dipyridamole concentration was increased to 50μM (Table 7.5). The disappearance of PRPP in the dipyridamole-treated cells was followed by an accumulation of pentose 5-phosphates (ribulose 5-phosphate, xylulose 5-phosphate and ribose 5-phosphate) (Table 7.6), indicating that the synthesis of PRPP is hampered under these conditions.

7.3 Discussion

In this chapter the mechanism of action of four potent inhibitors of nucleotide biosynthesis that display different pharmacological profiles as antitumour agents has been investigated. Earlier studies with antimetabolites that interfere with purine and pyrimidine nucleotide biosynthesis have focused mainly on their effects on cellular DNA and RNA synthesis. The present study has used four different inhibitors as model compounds to determine the relative impact of guanine and adenine nucleotide depletion on cellular differentiation and proliferation. To do this, we have compared the biochemical effects of total purine nucleotide depletion caused by acivicin with those caused by the specific depletion of guanine nucleotides by mycophenolic acid, an inhibitor of IMP dehydrogenase, and of adenine nucleotide depletion caused by alanosine, a metabolite which inhibits adenylosuccinate synthetase. This study has also made use of the effect of dipyridamole (a potent inhibitor of nucleoside and nucleic acid base transport) on purine and pyrimidine synthesis as well as its effect on cellular differentiation.

The depletion of purine nucleotide pools by mycophenolic acid and acivicin led to a degree of 'maturation' of HL60 cells ascertained by the enhanced production of reactive oxygen intermediates implied in higher rates of CL and cytochrome C reduction (Figs 7.3

Table 7.5 : PRPP and IMP levels in untreated and inhibitor-treated cells.

Treatment of HL60 cells with inhibitors	PRPP nmole/10^7cells	IMP nmole/10^7cells
None	0.40±0.01	154±6.4
Mycophenolic acid	0.60±0.02**	158±1.4
Acivicin	1.26±0.24**	ND
Alanosine	0.92±0.08**	82±2.95
Dipyridamole*	0.00	102±13
Dipyridamole#	0.15+0.03**	180±3

The concentration and duration of inhibitor treatment are described in the legend to Table 7. PRPP and IMP were measured as described in the Materials and Methods section. The values given are the mean ± S.E.M. of at least three separate experiments, each performed in triplicate. **$p<0.02$, significantly different from the corresponding value of control HL60 cells.

Table 7.6: Pentose 5-phosphates in untreated and dipyridamole-treated cells.

Cell type	Ribulose 5-phosphate (nmole/10^7cells)	Xylulose 5-phosphate (nmole/10^7cells)	Ribose 5-phosphate (nmole/10^7cells)
HL60	0.00	0.00	11±1
Dipyridamole (10μM)-treated cells	62±8	3.5±0	15±5**
Dipyridamole (50μM)-treated cells	106±16	4.4±0.5	15±2**

Pentose 5-phosphates were measured as described in the Materials and Methods section. The values given are the mean ± S.E.M. of six different readings from two separate experiments, each performed in triplicate. **$p<0.05$, significantly different from the corresponding value of control HL60 cells.

and 7.4). These changes were accompanied by a significant loss of proliferative capacity as assessed by 60-70% inhibition of [^{14}C]-thymidine uptake and 50% inhibition of [^{3}H]-uridine incorporation (Table 7). Underlying these changes were substantial decreases in the guanylate (90%) and adenylate (75-95%) pools revealed by [^{14}C]-formate incorporation (Table 7.1). To ascertain whether the induction of maturation was associated with the depletion of intracellular GTP, the GTP pool was restored by the simultaneous addition of guanosine or guanine to the mycophenolic acid-treated HL60 cells. These precursors prevented the depletion of GTP and, simultaneously, prevented the induction of myeloid maturation as evidenced by a decline in the luminol-dependent CL response and cytochrome C reduction (Figs 7.5 and 7.6). In contrast, the addition of exogenous adenine or adenosine to the growth medium had no effect on cellular maturation, while inosine or hypoxanthine partially reversed both the depletion of intracellular guanylate pools and the concomitant induction of myeloid maturation. The utilization of inosine and hypoxanthine for guanylate synthesis involves overlapping salvage and *de novo* purine nucleotide biosynthetic pathways, each of which ultimately proceeds through IMP, whereas the utilization of guanosine or guanine to form guanosine ribonucleotides involves the direct salvage of guanine to GMP, which bypasses the synthetic pathway from IMP. The results are in agreement with the observed decrease in the utilization of [^{14}C]-hypoxanthine, despite the enhanced activity of HGPRT. The utilization of [^{14}C]-hypoxanthine for guanylate synthesis, which proceeds through IMP, was decreased by 30% due to partial inhibition of IMP dehydrogenase and GMP synthetase in mycophenolic acid-treated cells (Table 7.2). Although these cells possessed the same adenylosuccinate synthetase activity as control HL60 cells, there was significant inhibition (15%) of the rate of synthesis of adenylates from [^{14}C]-hypoxanthine, possibly because of the depletion of the GTP pools which participate in the synthesis of adenosine ribonucleotides.

Simultaneous addition of exogenous guanosine or guanine with acivicin to the growth medium of undifferentiated HL60 cells caused only partial reversal of myeloid maturation

as assessed by the CL response and cytochrome C reduction (Figs 7.7 and 7.8). The incomplete reversal of maturation in this case may have been due to the depletion of CTP caused by the inhibition of CTP synthetase (Hindenburg *et al.*, 1985). Addition of both cytidine and guanosine are required to fully reverse the capacity of acivicin-treated cells to return to their normal proliferative state (Zhen *et al.*, 1983) and both may have been necessary to fully inhibit the myeloid maturation induced by acivicin in HL60 cells. Despite enhanced activity of HGPRT and a similar rate of uptake of [^{14}C]-hypoxanthine as that seen in control HL60 cells, guanylate synthesis in acivicin-treated cells was inhibited by 25%, possibly due to inhibition of GMP synthetase. The corresponding increase in the synthesis of adenylates (by 25%) might have occurred due to an acceleration of purine synthesis stimulated by the fall in ATP, which would reduce the intensity of the feed-back inhibition responsible for the original block in *de novo* synthesis (Ullman *et al.*, 1979).

Acivicin and mycophenolic acid both acted as potent inhibitors of amidophosphoribosyl transferase as seen by the fall in the activity of the enzyme (Table 7.2) possibly due to the effects of these inhibitors on its *de novo* synthesis. Inhibition at this step is primarily responsible for the diminished formation of purine nucleotides, a phenomenon also believed to be responsible for the toxicity of these drugs. The inhibition of amidophosphoribosyl transferase by mycophenolic acid may proceed as an indirect consequence of the accumulation of IMP and XMP in response to the inhibition of IMP dehydrogenase and GMP synthetase (Table 7.2). On the other hand, acivicin has been shown to inhibit amidophosphoribosyl transferase directly by alkylating a cysteinyl residue at the glutamine-binding site of the enzyme, since it acts as an antimetabolite of glutamine (Weber *et al.*, 1984). Measurement of the IMP levels in mycophenolic acid and acivicin-treated cells failed to detect any accumulation of IMP; increases (2-7 fold) in the level of hypoxanthine were observed instead (Table 7.1) which may result from the dephosphorylation of IMP to inosine, and the conversion of inosine to hypoxanthine by nucleoside phosphorylase. As well as the inhibition of purine nucleotide biosynthesis,

acivicin and mycophenolic acid also caused a 28-35% inhibition of the *de novo* synthesis of pyrimidine nucleotides (Table 7.4). This inhibition is most likely directed at the activities of the enzymes carbamoyl phosphate synthetase and CTP synthetase, which are targets for the action of acivicin. In mycophenolic acid-treated cells the inhibition of pyrimidine nucleotide biosynthesis possibly occurred in response to depletion of the ATP pool, since this nucleotide is a precursor molecule required by both the *de novo* and salvage pathways of pyrimidine nucleotide biosynthesis. The salvage pathway is entirely dependent on ATP. The depletion of the ATP pool may have also been one of the factors contributing to the inhibition of pyrimidine biosynthesis in acivicin-treated cells. The inhibition of both purine and pyrimidine pathways in acivicin and mycophenolic acid-treated cells caused a 1.5 to 3-fold accumulation in the steady-state concentration of PRPP (Table 7.5).

A general phenomenon has been observed with many inhibitors of nucleotide biosynthesis: the potent inhibition of either the *de novo* pyrimidine or purine nucleotide pathway is accompanied by a 'complementary stimulation' of flux through the unaffected pathways (Lyons, 1989). Blockade of the *de novo* pyrimidine nucleotide pathway by dichloroallyl lawsone (Kemp *et al.*, 1986) or pyrazofurin (Sant *et al.*, 1989), for example, leads to complementary stimulation of purine nucleotide biosynthesis. In a similar way, studies with murine leukaemic cells L1210 have shown that the depletion of intracellular guanylate pools by incubation with glutamine antimetabolites for 24 hours causes minor depletion of ATP pools and complementary stimulation of pyrimidine nucleotide biosynthesis due to a 'sparing' action of the inhibition of purine synthesis on glutamine and PRPP concentrations (Lyons, 1990). In the case of HL60 cells, the phenomenon of complementary stimulation was not observed, possibly because the longer periods of exposure of the cells to inhibitors (3 to 4 days) led to substantial depletion of ATP and, hence, to inhibition of pyrimidine nucleotide biosynthesis.

Treatment of HL60 cells with alanosine had no effect on cellular maturation (Figs 7.3 and 7.4) even though cellular proliferation was arrested by 62% (Table 7). [^{14}C]-Formate

labelling experiments showed that total purine nucleotide biosynthesis *de novo* was inhibited by 55%, although this was more severe (82%) in the case of the adenylates than the guanylates (63%) (Table 7.1). The inhibition of both adenylate and guanylate synthesis was most likely due to inhibition of the enzymes amidophosphoribosyl transferase, adenylosuccinate synthetase and IMP dehydrogenase (Table 7.2). The possible inhibition of amidophosphoribosyl transferase due to accumulation of IMP is consistent with the five-fold higher concentration of hypoxanthine in these cells compared with untreated controls (Table 7.1). Even though no adenylosuccinate synthetase activity could be detected in the isolated cells, [^{14}C]-hypoxanthine labelling showed that these cells were indeed capable of synthesizing 20% of their adenylates via IMP (Table 7.3). The activity of HGPRT was enhanced by 30% and there was a corresponding decrease (85%) in the activity of APRT (Table 7.2). Besides the inhibition of purine nucleotide biosynthesis there was a 25% inhibition of *de novo* pyrimidine nucleotide biosynthesis (Table 7.4), which may have occurred due to the inhibition of aspartate transcarbamoylase (Gale and Schmidt, 1968). The combined inhibition of both pyrimidine and purine nucleotide biosynthesis caused an increase in the steady-state concentrations of PRPP by approximately 2.5-fold (Table 7.5). Similar findings have been observed in mutant lymphoma cells of the AU-100 line in which the adenylosuccinate synthetase activity was reduced to 20% (Ullman *et al.*, 1980).

Treatment of HL60 cells with dipyridamole (10μM) had no significant effect on cellular maturation in spite of a 48% inhibition of cellular proliferation (Table 7). Concentrations of dipyridamole greater than 20μM introduced a further complication – cytotoxicity – as monitored by a decrease in the percentage of cells excluding trypan blue ; at 50μM dipyridamole the viability of the cells was less than 50%. Dipyridamole (10μM) had no effect on the uptake of [^{14}C]-hypoxanthine by undifferentiated HL60 cells (Table 7.3), although it had substantial effects on *de novo* purine nucleotide biosynthesis (50% inhibition) (Table 7.1), perhaps due to inhibition of amidophosphoribosyl transferase (Table 7.2) by the increased intracellular concentration of adenosine. Adenosine is

generated inside the cell through the action of 5'-nucleotidase on AMP derived from the adenosine nucleotide pool. In normal conditions all of the adenosine formed is removed rapidly by the action of either adenosine deaminase (with subsequent metabolism to inosine) or adenosine kinase (which reintroduces it into the metabolic cycle leading to adenine nucleotide production) (Schrader *et al.*, 1972). Dipyridamole is a potent inhibitor of the cellular uptake of adenosine (Harker and Kadatz, 1983; Arch and Newsholme, 1978) and in its presence the half-life of adenosine is prolonged strikingly in red blood cells (Klabunde, 1983) leading to the accumulation of adenosine. The accumulated adenosine would either be phosphorylated by adenosine kinase or deaminated by adenosine deaminase: since the K_m of adenosine kinase is significantly lower than that of adenosine deaminase, the probability is that the majority of the accumulated adenosine would be phosphorylated to AMP. This product would exert its inhibitory effect on amidophosphoribosyl transferase and slow the rate of *de novo* purine nucleotide biosynthesis. Dipyridamole also inhibited *de novo* pyrimidine nucleotide biosynthesis (Table 7.4), possibly as a result of ATP depletion caused by inhibition of purine nucleotide biosynthesis. Lack of PRPP (Table 7.5) in the cells treated with low concentrations of dipyridamole (10μM) may be one of the factors contributing to the inhibition of both purine and pyrimidine nucleotide biosynthesis. PRPP depletion in these cells is probably the net result of its enhanced rate of utilization (by pathways other than purine and pyrimidine biosynthesis) exceeding its rate of synthesis. The slightly higher demand for PRPP may be imposed by the 5% enhancement in the salvage pathway of purine biosynthesis (Table 7.3). The 46% enhancement in the activity of PRPP synthetase (Table 7.2) is consistent with the higher demand for this precursor by dipyridamole (10μM)-treated cells. Moreover, inhibition of amidophosphoribosyl transferase would cause depletion of aminoimidazole ribosyl 5-phosphate, a precursor needed for the synthesis of the pyrimidine moiety of thiamine pyrophosphate (Henderson, 1972). Deficiency of thiamine pyrophosphate would inhibit transketolase, a key regulatory enzyme of the non-oxidative pentose phosphate pathway. As a result, the activity of the non-oxidative pentose phosphate pathway would be hampered, possibly

resulting in the partial/complete inhibition of the synthesis of ribose 5-phosphate and accumulation of xylulose 5-phosphate and ribulose 5-phosphate (Table 7.6) respectively. Even though the overall steady-state concentration of ribose 5-phosphate was elevated by 30% compared with the control HL60 cells, the lack of PRPP in dipyridamole-treated cells suggests that the particular pool of ribose 5-phosphate responsible for this increase may not be available for PRPP synthesis, possibly because of its inappropriate compartmentation. The accumulated ribose 5-phosphate may be one of the end-products of adenosine metabolism (Fig 7.9).

Amidophosphoribosyl transferase was also inhibited in both acivicin and alanosine-treated cells, but these cells accumulated PRPP instead of depleting it as occurred in dipyridamole-treated cells. The accumulation of PRPP may have been a consequence of the inhibition of purine and pyrimidine nucleotide biosynthesis or in response to a lack of demand for PRPP by other pathways. Low levels of PRPP (37% of the control cells) were detected in cells treated with higher concentrations of dipyridamole (50μM).

Even though the various actions of dipyridamole (as a coronary vasodilator, antithrombic drug and inhibitor of nucleoside and nucleic acid base transport) are known, this is the first report where its action as an inhibitor of both *de novo* purine and pyrimidine nucleotide biosynthesis has been demonstrated. Further experiments are needed to elucidate experimentally the mechanism of inhibition of amidophosphoribosyl transferase and transketolase. This drug can be used in cancer chemotherapy as an inhibitor of *de novo* purine and pyrimidine nucleotide biosynthesis on the basis of its effects on nucleoside and nucleic acid base transport.

The depletion of intracellular guanylate pools in HL60 leukaemic cells by a variety of nucleotide biosynthesis inhibitors has been associated recently with the regulation of differentiation of these cells (Lucas *et al.*, 1983; Sokoloski and Sartorelli, 1987; Sokoloski *et al.*, 1986; Wright, 1987; Knight *et al.*, 1987) suggesting that an analogous reduction of intracellular guanylate levels by acivicin and mycophenolic acid might

promote the induction of differentiation of promyelocytic HL60 cells to a more mature phenotype. These findings are supported by the failure of guanosine to prevent the induction of differentiation of HL60/HGPRT$^-$ cells (i.e. those which lack the enzyme HGPRT) by mycophenolic acid, and are consistent with the action of guanosine in preventing differentiation of control HL60 cells being due to its conversion to guanine nucleotides (Sokoloski *et al.*, 1986). Moreover, the myeloid leukaemic cell line RDFD2 has been shown to be resistant to the induction of maturation when the inducing agents were RA and dimethylformamide (Knight *et al.*, 1987). These cells failed to show decreased IMP dehydrogenase activity and reduced guanylate concentration on exposure to the inducing agents. Collectively, these findings demonstrate that guanylate nucleotides play an important role in cellular differentiation.

Exposure of HL60 cells to acivicin and mycophenolic acid caused a pronounced reduction, within three to four days, in the levels of intracellular pools of both GTP and ATP . The reduction in the level of ATP is probably an indirect effect of the reduction of GTP as it is observed only when HL60 cells are exposed to inhibitors for periods greater than 24 hours. The exposure of HL60 cells to tiazofurin, an inhibitor of IMP dehydrogenase, for only four hours resulted in a substantial decrease in the steady-state concentration of intracellular GTP while the ATP pool remained intact and these conditions supported the induction of myeloid maturation (Wright, 1987).

The regulatory steps controlled by intracellular purine nucleotide triphosphates that might participate in triggering the differentiation of HL60 cells to a mature phenotype remain unknown. GTP is associated with a wide variety of cellular processes involved in cellular replication and differentiation. GTP-binding proteins, for example, are known to regulate a variety of critical cellular activities, including the ribosomal assembly of polypeptides and receptor-mediated responses to extracellular stimuli (Maitra *et al.*, 1982; Rodbell, 1980; Hughes, 1983). GTP-binding proteins are also important in the regulation of hormone-sensitive adenylate cyclase. It is possible that the depletion of intracellular guanylates in HL60 cells may affect their ability to respond to proliferative

signals, thereby triggering cellular maturation as a permissive event. DNA synthesis in particular appears to be a specific target of guanine nucleotide depletion (Cohen and Sadee, 1983). Inhibition of DNA synthesis is not usually the direct result of insufficient deoxyribonucleotide substrate but is dependent on the interruption of a process which uses either GTP or some other nucleotide (other than dGTP) derived from the guanine ribonucleotide pool as a cofactor (Cohen and Sadee, 1983).

GTP is known also to play a central role in the glycosylation of proteins containing N-linked oligosaccharide chains and its depletion interferes with the activation of both fucosyl and mannosyl units to their corresponding GDP-nucleotide sugars. This would reduce their incorporation into both glycoproteins and lipid-linked oligosaccharides. A role for glycoproteins in the induction of maturation of HL60 cells has been inferred from the use of anthracycline antibiotics that depress glycoprotein synthesis (Morin and Sartorelli, 1984). Tunicamycin, a potent inhibitor of glycoprotein synthesis, is capable of inducing differentiation of HL60 cells (Nakayasu *et al.*, 1980) and acivicin depletes CTP and GTP pools and curtails membrane resialylation (Hindenburg, 1985). Cytidine and guanosine must both be added to restore sialic acid synthesis from precursor sugars. Hence, GTP depletion caused by acivicin and mycophenolic acid in HL60 cells might cause changes in the surface membrane that are involved in the induction of maturation. As alterations in cell membrane properties can affect the behaviour of neoplastic cells, treatment with agents like acivicin and mycophenolic acid may exert antineoplastic and maturational effects through altered expression or defects in the properties, of membrane macromolecules, in addition to inhibition of DNA synthesis and other growth-related pathways.

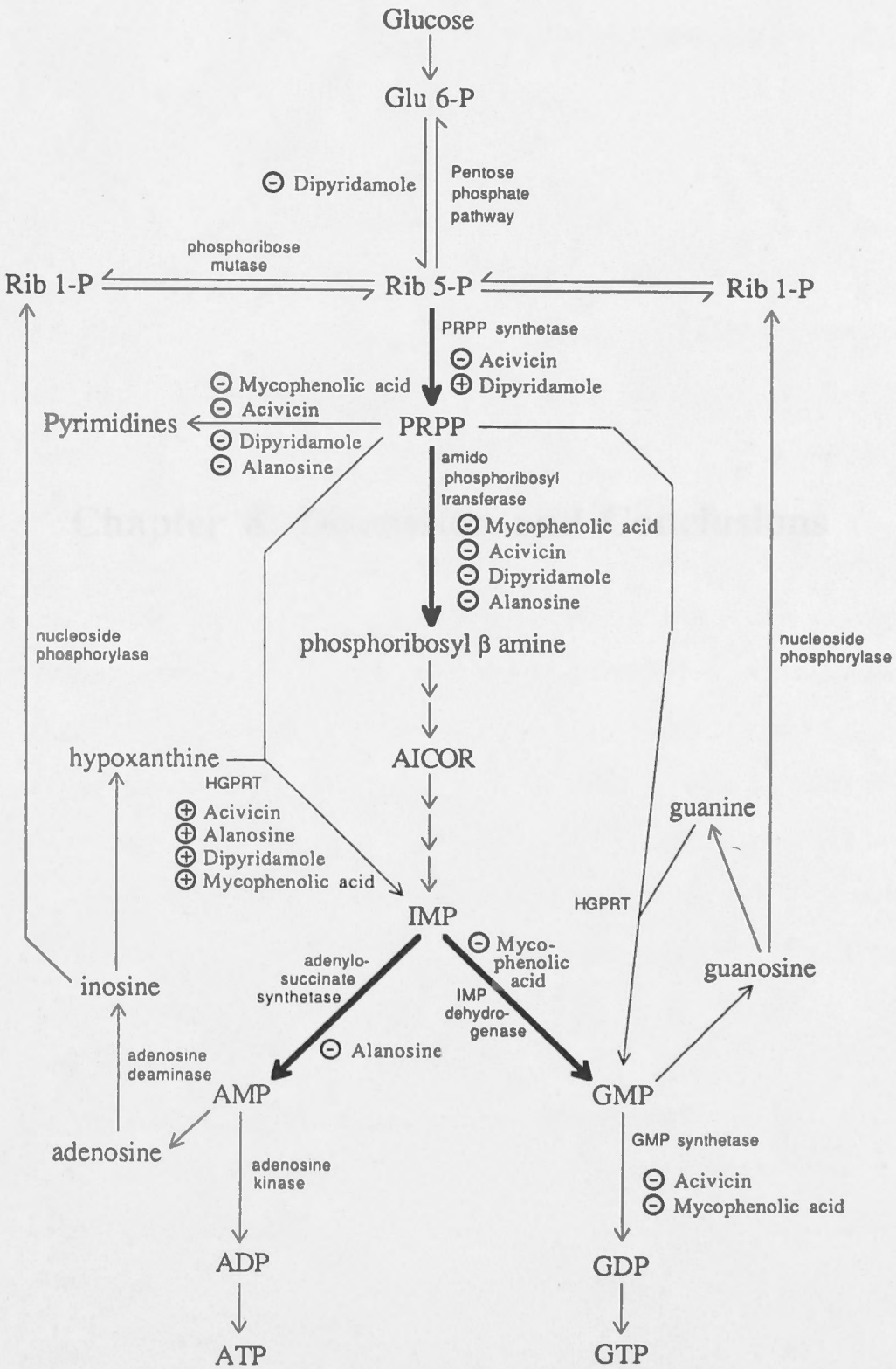
The treatment of HL60 cells with alanosine and dipyridamole separately resulted in guanylate depletion, but this effect was secondary to the depletion of the adenylates and required prolonged exposure of the cells to the inhibitors. We propose that the depletion of guanine ribonucleotides resulting from the inhibition of early *de novo* purine biosynthesis (as in the case of acivicin treatment) or due to the specific inhibition of *de*

novo guanine nucleotide biosynthesis (as in the case of mycophenolic acid treatment) may play a significant role in initiating the complex mechanism of differentiation in HL60 cells. Possible mechanisms of action of the inhibitors on purine and pyrimidine nucleotide biosynthesis in HL60 cells are shown schematically in Fig 7.9.

Fig 7.9: Possible mechanism of action of mycophenolic acid, acivicin, alanosine and dipyridamole on purine and pyrimidine biosynthetic pathways in HL60 cells.

Dark arrows indicate the sites which are enhanced in HL60 cells. (-) indicates sites of inhibition exerted by inhibitors on the purine and pyrimidine biosynthetic pathways in HL60 cells.

Fig 7.9


Glucose
Glu 6-P
⊖ Dipyridamole
Pentose phosphate pathway
phosphoribose mutase
Rib 1-P
Rib 5-P
Rib 1-P
PRPP synthetase
⊖ Acivicin
⊕ Dipyridamole
⊖ Mycophenolic acid
⊖ Acivicin
Pyrimidines
PRPP
⊖ Dipyridamole
⊖ Alanosine
amido phosphoribosyl transferase
⊖ Mycophenolic acid
⊖ Acivicin
⊖ Dipyridamole
⊖ Alanosine
nucleoside phosphorylase
phosphoribosyl β amine
nucleoside phosphorylase
hypoxanthine
AICOR
HGPRT
⊕ Acivicin
⊕ Alanosine
⊕ Dipyridamole
⊕ Mycophenolic acid
guanine
HGPRT
IMP
⊖ Myco-phenolic acid
adenylo-succinate synthetase
IMP dehydro-genase
inosine
guanosine
⊖ Alanosine
adenosine deaminase
AMP
GMP
adenosine
adenosine kinase
GMP synthetase
⊖ Acivicin
⊖ Mycophenolic acid
ADP
GDP
ATP
GTP

Chapter 8: Discussion and Conclusions

Many cell lines – including embryonic and haematopoietic – which can be induced to differentiate in culture have been used as model systems for the study of differentiation (Marks and Rifkind, 1978; Harris and Ralph, 1985). Study of the processes which occur during induced differentiation of these cell lines sheds light not only on the particular events leading to terminal differentiation but also provides an opportunity to establish general guidelines which may apply to other systems.

There are certain advantages and disadvantages in the use of cell lines for the study of differentiation. A major advantage is that they provide a relatively homogenous cell population, a condition not available *in vivo*. If synchrony can be achieved, the differentiating process can be monitored accurately in homogenous lines that are free from the background contamination contributed by cells at vastly different stages of differentiation. Cell lines which differentiate upon induction also offer the opportunity to study the process of 'commitment to differentiation', since variant lines which are resistant to differentiation are often available as controls (Leftwich, 1987). Futhermore, the availability of a large spectrum of material of diverse genetic origin provides an opportunity to study the processes that regulate differentiation at the molecular level. The major disadvantage is that adaptation to tissue culture conditions sometimes results in aberrant gene expression, making it uncertain whether the processes under study are equivalent to those that prevail *in vivo*. Moreover, the maintenance of cells in culture for long periods also results in a population which may vary significantly from the initial cell isolate. The longer the cells are maintained in culture the greater the likelihood that genotypic variation will arise. Care has to be taken when comparing results from different laboratories or when extrapolating from *in vitro* systems to those which occur *in vivo* (Leglise *et al.*, 1988). The inducing agents used to initiate differentiation of cell lines are often non-physiological reagents, such as DMSO, or analogues of intracellular 'second messengers,' such as PMA, that are used at concentrations far greater than those of their physiological counterparts *in vivo*. It is unknown if the induction of differentiation by particular chemical agents occurs by a similar mechanism or pattern of gene

expression to that found *in vivo*. Many cell lines are immortal, with unlimited proliferative capacity, able to survive in culture for an indefinite period. During the induction of differentiation, cell lines become committed to a specific course of differentiation. This may be followed by the cessation of proliferation and a concomitant switch from immortality to mortality. Many of the changes in gene expression (e.g. in the expression of oncogenes like *c-myc*, *c-myb*, *c-fos*, etc) may reflect this shift from immortality to mortality rather than changes specific to the differentiation process (Birnie, 1988). Changes in gene expression which regulate cessation of proliferation could be considered as secondary to the differentiation process itself.

In the present study, the HL60 cell line has been used as a model system to elucidate the phenotypic and biochemical events which accompany terminal differentiation. In this system changes in the uninduced cells can be used as controls to pinpoint those characteristics which are specific for differentiation. As these cells are multipotent, they also provide an opportunity to investigate the mechanisms that govern the differentiation of progenitor cells along different cellular pathways (Fontana *et al.*, 1981; Fischkoff *et al.*, 1984; Yoneda *et al.*, 1991). This provides interesting possibilities for the study of 'commitment to differentiation', since gene expression patterns identified in one lineage may reveal regulatory mechanisms that are common to other differentiation pathways.

The HL60 cell line is also a useful tool for the study of 'maturation arrest' in leukaemic disorders as it provides an insight into the nature of the leukaemic lesion. It is interesting to note that drugs which are most effective in the treatment of patients with acute myelogenous leukaemia, such as anthracyclines, are also inducers of HL60 cell differentiation *in vitro* (Schwartz and Sartorelli, 1981). Hence, it can be speculated that the effectiveness of these drugs may be due in part to their ability to promote leukaemic cell differentiation as well as to their overtly cytocidal effect on malignant cells.

Following differentiation of HL60 cells with dimethylsulphoxide, butyrate and PMA, these cells acquire some of the morphological and functional characteristics of normal granulocytes, monocytes and macrophages (Chapters 2 and 3). Since differentiating HL60 cells have been shown to undergo many of the phenotypic changes known to occur during myeloid differentiation from promyelocytes to granulocytes (Collins *et al.*, 1978; Breitman *et al.*, 1980), many of the programmed changes in gene expression which occur during myelopoiesis and give rise to the fully-differentiated phenotype, must also take place during HL60 cell differentiation despite the fact that they are starting out as transformed cells. Results from different laboratories have shown that, even though a large proportion of the chemically-induced HL60 cells resemble their normal mature cell counterparts present in peripheral blood, a number of their phenotypic and biochemical properties remain unaltered, or only partially unaltered, after differentiation. This might be attributed to the heterogeneity of the original HL60 cell population and a lack of synchrony in the progress of individual cells through the cell cycle at the time of addition of the inducing agent, resulting in variation in the timing of the appearance of the differentiated phenotype in individual cells. The possibility that a subpopulation of the cells is refractory to the differentiating action of the particular inducer might also contribute to the incomplete differentiation. Skubitz *et al.* (1982) studied changes in ^{125}I-labelled surface antigens after induction of HL60 cells with RA. They reported that, although surface antigens characteristic of mature granulocytes could be detected in chemically-induced HL60 cells, they represented a smaller proportion of the total population of cell-surface antigens than those found in normal fully-differentiated granulocytes. Yokata *et al.* (1984) have demonstrated critical differences in polypeptide synthesis during granulocytic differentiation that are indicative, when compared with the protein pattern obained with normal cells, of partial or/ incomplete differentiation of HL60 cells. In a much earlier study, Gallagher *et al.* (1979) reported that DMSO-induced HL60 cells fail to synthesize two markers of mature granulocytes (lactoferrin and alkaline phosphatase), which also suggests that HL60 differentiation by DMSO is not complete. Ferrero *et al.* (1983) reported that, although 90% of PMA-induced HL60 cells resembled

human macrophages, in that they possessed some myelomonocytic cell markers and expressed a number of membrane-bound proteins synthesized predominantly by human macrophages (Todd *et al.*, 1981; Feuerstein and Cooper, 1984; Yokata *et al.*, 1984), these cells failed to produce reactive oxygen intermediates upon appropriate stimulation (Newburger *et al.*, 1981). Therefore, although chemically-induced HL60 cells may not be completely identical to their normal cellular counterparts, the available evidence suggests that they are granulocyte/monocyte/macrophage-like in at least some important respects.

The work presented in this thesis confirms a number of findings cited in the literature. HL60 cells differentiated with dimethylsulphoxide, butyrate and PMA lack many of the important functions of normal mononuclear and polymorphonuclear phagocytes. In spite of producing reactive oxygen intermediates in response to PMA at a similar rate to that observed in normal neutrophils and monocytes, DMSO and butyrate-induced cells release abnormally low amounts of lysosomal enzymes. This may be the result of a defective degranulation process or due to the failure of the differentiating stimulus to increase the synthesis of specific granular constituents. This deficit is consistent with the lack of a vigorous phagocytic function in these cells, characterized by a very weak CL response in response to opsonized *S.aureus* compared with that of mature circulating phagocytes. The lack of myeloperoxidase activity (assessed by direct enzymic assay and demonstrated functionally by a low rate of CL in response to PMA in the absence of HRP), and the failure of the majority of the DMSO-differentiated cells to shift to a higher density on Percoll gradients, further indicates that the normal maturation process is not complete upon differentiation. Even though the PMA-induced HL60 cells resembled human macrophages morphologically, they had lower levels of lysosomal enzymes and failed to produce reactive oxygen intermediates in response to PMA.

The generation of reactive nitrogen intermediates (nitric oxide) by endothelial cells, polymorphonuclear leukocytes and mononuclear phagocytes has been well characterized (Ignarro, 1990; McCall *et al.*, 1989; Nathan and Hibbs, 1991). Although both endothelial

cells and macrophages are known to regulate the differentiation of haemopoietic cells in the bone marrow by secreting a variety of interleukins, colony stimulating factors and other cytokines (Greenberger, 1991), the possible differentiating effects of nitric oxide produced by these cells is not known. Since treatment of HL60 cells with DMSO produces neutrophil-like cells, it was considered important to evaluate their capacity for nitric oxide production in order to determine whether this correlates with cellular differentiation. Measurement of reactive nitrogen intermediates produced in the presence of L-arginine in reponse to PMA showed that approximately the same amount of nitrate was produced by DMSO-differentiated cells as by PMNs separated from human blood. L-NMMA inhibited the production of nitrate in both cell types, indicating that the nitrate was derived from nitric oxide by the action of nitric oxide synthase on L-arginine. The basal level of nitrate in human PMNs was eight-fold higher than that measured in DMSO-differentiated cells, which may correlate positively with the vigorous phagocytic activity of human PMNs and its absence in DMSO-differentiated cells. Magrinat *et al.* (1992) have reported recently the induction of differentiation of HL60 cells by nitric oxide, as a consequence of which there were marked increases in mRNA for tumour necrosis factor-α and interleukin $-1\ \beta$ and corresponding decreases in the expression of *c-myc* and *c-myb* oncogenes. Earlier studies had shown that drugs capable of *releasing* nitric oxide induced differentiation and elevated the level of cGMP in HL60 cells, the degree of differentiation correlating roughly with the cellular level of cGMP (Boss, 1989). Nitric oxide is also known to inhibit cellular proliferation in various cell lines (Albina *et al.*, 1991; Lepoivre *et al.*, 1991). These studies suggest that nitric oxide may participate in the regulation of cellular differentiation. The lack of significant nitric oxide production above the basal level by HL60 cells [as evidenced by the small increment (6%) in the nitrate levels of cells responding to PMA in the presence of L-arginine] may be one facet of the processes responsible for their continuous proliferation and inability to undergo differentiation. The basal level of nitric oxide in HL60 cells may be necessary to sustain proliferation, as has been observed in other tumour cell lines (Efron *et al.*, 1991). Simultaneous measurements carried out on the formation of reactive nitrogen and oxygen intermediates in response to PMA

showed that undifferentiated HL60 cells had an enhanced rate of CL and a decreased rate of cytochrome C reduction in the presence of increasing concentrations of L-arginine. The enhanced CL response was inhibited and the decreased cytochrome C reduction was enhanced by L-NMMA in a concentration-dependent manner, indicating that the small amount of nitric oxide produced by undifferentiated cells in addition to that present basally may still play some part in the production of ROI. In contrast, both DMSO-differentiated cells and human PMNs responded to PMA with a decreased CL response and decreased cytochrome C reduction when increasing concentrations of L-arginine were added. To some extent these inhibitions were relieved by L-NMMA in a similar dose-dependent manner. The inhibition of superoxide anion production (as assessed by cytochrome C reduction) by L-arginine in HL60 and DMSO-treated cells has also been reported for activated macrophages (Albina *et al.*, 1989; Rubanyi *et al.*, 1991) and human PMNs (McCall *et al.*, 1989; Wright *et al.*, 1989) and may be indicative of a cytoprotective action of nitric oxide towards the target cells. However, the current work is the first report in which the enhancement and /or inhibition of luminol CL response by L-arginine has been demonstrated in HL60, DMSO-treated cells and human PMNs. A possible role for the peroxynitrite anion has been proposed to explain the enhancement and inhibition of luminol CL in the three different cell types. In HL60 cells, the putative peroxynitrite anion may appear after a lag period of 5 mins, when the levels of reactive oxygen intermediates (especially the superoxide anion) have fallen and the concentration of nitric oxide synthesized exceeds that of the reactive oxygen intermediates. The formation of the peroxynitrite anion under these conditions might then lead to the production of strong oxidants with the capacity to amplify the luminol CL response. On the other hand, in DMSO-treated cells and human PMNs, the production of nitric oxide by L-arginine overlaps in time with the production of reactive oxygen intermediates. The rate of superoxide anion produced by these cells in response to PMA would maintain a large excess of reactive oxygen intermediates compared with the concentration of nitric oxide, leading to possible inhibition of the formation of the peroxynitrite anion. Coupled to this phenomenon is the inhibition of superoxide anion formation by L-arginine, which curtails the luminol-

dependent CL responses drastically. Hence, it has been proposed that the formation of precursors that lead to peroxynitrite anion formation may be responsible for the enhancement of the luminol-dependent CL response in cells such as HL60, which have a very low capacity for reactive oxygen intermediate formation, while in cells which produce elevated levels of reactive oxygen intermediates, the formation of peroxynitrite anion is inhibited, leading to the overall inhibition of the luminol CL response. This proposal is in agreement with the enhancement of the luminol CL response in isolated hepatocytes treated with L-arginine (Wang *et al.*, 1991), which presumably produce ROI in much smaller amounts than PMNs; and the production of ROI at rates three orders of magnitude higher than those of nitric oxide in dibutyryl cAMP-differentiated HL60 cells and human PMNs in response to a chemotactic peptide (Schmidt *et al.*, 1988).

Acquisition of phenotypic characteristics that are similar to those of their blood cell counterparts provides evidence of the onset of differentiation in chemically-differentiated cells, but to verify the degree of actual maturation achieved it is important to use an animal model to confirm the corresponding change in tumourigenicity/leukamogenicity. For this purpose, animal experiments were designed involving the transplantation of both leukaemic and chemically-differentiated HL60 cells into nude mice. Both cell types resulted in the development of tumours, suggesting once again that differentiation was defective or incomplete. When the cell number injected was reduced to values as low as 3×10^6, however, the incidence of tumours declined and the survival time of the mice bearing tumours of differentiated cell origin was increased significantly. The formation of tumours by differentiated cells can be attributed to the presence of a threshold population of residual differentiation-resistant cells present in the original HL60 cell line (Leftwich, 1987). These observations are consistent with those of Lozzio and Machado (1982), who showed that treatment of leukaemic K562 cells with 2% DMSO caused a significant loss of malignancy as determined by their capacity to develop myelosarcomas in lasat mice. The pattern of tumour growth was different when the site of transplantation was changed: subcutaneous

transplantation produced massive tumours restricted to the site of injection, while intraperitoneal injection was followed by the development of ascites and the appearance of small solid tumours in the peritoneal cavity just above the bladder. This result shows that the pattern of tumour growth is influenced by the site of transplantation, due perhaps to transient changes associated with the establishment of the cells in a different microenvironment (Weiss, 1980). No distant metastases were observed in any of the dead mice. In some cases the tumours displayed green coloration, possibly attributable to the release of myeloperoxidase from neutrophils accumulating at the site of injection, consistent with the observations reported by Potter *et al.* (1985). Morphological examination of tumour cells recovered from the nude mice showed strong resemblance to cultured HL60 cells, yet more detailed study of their phenotypic characteristics showed them to be quite different from their parental cells, consistent with the acquisition of new properties. A higher rate of respiratory burst activity in HL60/T cells compared with 9DMSO/T, 9BUT/T and cultured HL60 cells may reflect the presence of natural biological factors (such as CSF's) in the host tissue, which can affect the differentiation of cells proliferating *in vivo*. The lack of such differentiation in 9DMSO/T and 9BUT/T cells may reflect a greater requirement for the differentiating factors than HL60/T cells which may exceed the concentration the host tissue is capable of producing. Moreover, DMSO, butyrate and PMA-induced cells failed to produce differentiated tumour cells to the same extent as the undifferentiated HL60 cells, which emphasizes the different interaction that differentiated and undifferentiated cells have with the host environment *in vivo*. These observations are consistent with those of Machado *et al.* (1984), who reported the differentiation of the leukaemic KG-1a sub-line when cells were transplanted subcutaneously into new-born nude mice. Moreover, Fogh and Hajdu (1975) observed that, of the large series of tumours transplanted directly into nude mice from surgical specimens, only 25% showed differentiated properties while the rest had karyotypic and phenotypic properties similar to those of the parental cells.

Although much is known about the morphological and phenotypic changes which accompany differentiation of HL60 cells, very little is known about the corresponding pattern of substrate utilization. Hence the metabolic fates of two major fuels, glucose and glutamine, that are used by undifferentiated HL60 and DMSO-differentiated cells were evaluated. Surprisingly, there was no significant difference in the rate of utilization of exogenous glucose between HL60 and DMSO-differentiated HL60 cells. The activities of the glycolytic enzymes were similarly unaffected. In contrast, the activity of the oxidative segment of the pentose phosphate pathway was enhanced selectively by differentiation as evidenced by the increased activities of glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and increased oxidation of [1-^{14}C]-glucose relative to [6-^{14}C]-glucose by DMSO-treated cells. These results are consistent with those of Newburger *et al.* (1979). Enhanced oxidation of glucose by this pathway would maintain the NADP/NADPH couple in a highly reduced state in anticipation of the respiratory burst activity that is a characteristic function of polymorphonuclear leukocytes. The enzymes of the non-oxidative pentose phosphate pathway remained unaffected by differentiation, consistent with the absence of any change of emphasis in the forward and reverse reactions of the non-oxidative limb of the pentose phosphate pathway. The quantitative contribution of the F-type pentose cycle to glucose metabolism was insignificant (<0.01%), while the presence of an alternative L-type pentose phosphate pathway has been inferred in both HL60 and DMSO-differentiated cells, although the evidence for this conclusion is incomplete. Addition of exogenous glutamine to the medium as a co-substrate depressed glucose utilization in both HL60 and DMSO-treated cells, possibly because of allosteric inhibition of phosphofructokinase, the rate-limiting step in glycolysis. The consequent accumulation of hexose 6-phosphates would divert the flux of glucose towards the synthesis of glycogen and the oxidative segment of the pentose phosphate pathway, making the cells more functionally ready to meet their metabolic demands. These observations are consistent with those reported for colonocytes (Ardawi and Newsholme, 1985) and human diploid fibroblasts (Zielke *et al.*, 1975) where the presence of glutamine inhibited glucose utilization as a consequence of the inhibition of

phosphofructokinase. In contrast, in enterocytes and lymphocytes (Watford *et al.*, 1979; Ardawi and Newsholme, 1983) glutamine increased the utilization of glucose by *enhancing* the activity of phosphofructokinase.

In spite of a two-fold higher maximum catalytic activity of glutaminase, undifferentiated HL60 cells utilized glutamine as a sole substrate at half the rate of the differentiated cells. The lower rate of glutaminolysis by undifferentiated cells may be attributable to the limitation of glutamine transport across the mitochondrial inner membrane, possibly due to low intrinsic activity of the Na^+/H^+ exchanger at the plasma membrane (Besterman and Cautrecasas, 1984). Differentiation of HL60 cells by DMSO has been shown to activate the exchanger (Restrepo *et al.*, 1987), which may be responsible for the greater overall rate of glutamine utilization by these cells in spite of lower glutaminase activity. Similarly, the stimulation of glutaminolysis by glucose may have occurred because of activation of the mitochondrial glutamine transporter in response to the activation of the Na^+/H^+ exchanger by the acid equivalents produced by the operation of the oxidative pentose phosphate pathway upon addition of glucose (Restrepo *et al.*, 1987).

A large proportion of the glutamine utilized by both HL60 and DMSO-differentiated cells accumulated as glutamate, aspartate and alanine, while approximately 35% was oxidized to CO_2. The high proportion of glutamine oxidized by these cells is attributable to the presence of mitochondrial NAD(P)-linked malic enzyme (Dworkin and Dworkin-Rastl, 1991) and the further oxidation, via mitochondrial pyruvate dehydrogenase, of the pyruvate produced. Hence, NAD(P)-linked malic enzyme is likely to be an essential component of glutamine metabolism in both HL60 and DMSO-treated cells. This reaction is not only involved in the provision of energy, but also serves as a regulatory enzyme in generating macromolecule precursors at a sufficient rate to meet the extreme anabolic demands of proliferation. In both cell types the accumulation of glutamate, aspartate and alanine were decreased significantly when glucose was present in addition to glutamine. In rapidly-proliferating undifferentiated cells these observations may correlate with the increased utilization of these amino acids for

the synthesis of purines, pyrimidines and protein when both glucose and glutamine were present. Similar observations have been reported for rapidly-proliferating rat thymocytes (Brand, 1985; Brand *et al.*, 1987) where a low rate of aspartate formation from glutamine was observed in the presence of glucose. In both HL60 and DMSO-differentiated cells, nearly all of the glucose utilized was converted to lactate and very little was oxidized to CO_2. This is consistent with the inhibition of pyruvate oxidation (Eigenbrodt *et al.*, 1985) if the extramitochondrial pyruvate generated from glucose via the glycolytic pathway is not transported efficiently to the site of mitochondrial pyruvate dehydrogenase but reduced, instead, to lactate (Dworkin and Dworkin-Rastl, 1991).

Terminal differentiation is accompanied by the inhibition of cellular proliferation and a corresponding cessation of nucleotide biosynthesis. Since the increased concentrations of purine and pyrimidine nucleotides and deoxynucleotides are linked to neoplastic 'transformation and progression' (Weber, 1983), it was considered important to evaluate the rate of nucleotide biosynthesis in both HL60 cells and those induced to mature by DMSO. Studies on purine and pyrimidine metabolism showed that the maximum catalytic capacity of PRPP synthetase and the concentration of ribose 5-phosphate were higher in undifferentiated than in DMSO-differentiated cells, yet the steady-state concentration of PRPP remained unchanged. This suggests that there is greater utilization of PRPP in undifferentiated cells, which is consistent with rates of *de novo* purine and pyrimidine nucleotide synthesis that are six-fold and three-fold higher. This conclusion is supported further by significantly higher activities of the key regulatory enzymes of *de novo* purine nucleotide synthesis in HL60 cells. These observations are consistent with those of Lucas *et al.* (1983), who observed significant decreases in the purine nucleotide levels when HL60 cells were differentiated by dimethylformamide, RA and hypoxanthine. Friend and Reem (1975) observed similar changes on the differentiation of Friend erythroleukaemic cells by DMSO. The loss of control of *de novo* purine nucleotide synthesis in undifferentiated cells may occur because of a higher rate of recycling of purine nucleotides via the purine-ribose cycle, which might

enable the undifferentiated cells to maintain a sufficiently low steady-state concentration of purine nucleotides to avoid feed-back inhibition of PRPP synthetase and/or amidophosphoribosyl transferase.

There was an active purine salvage pathway in HL60 cells which remained unaltered by DMSO differentiation. The activities of the salvage enzymes were also the same before and after differentiation, which is consistent with the findings of Lucas *et al.* 1983 and Friend and Reem (1975). This pathway is the major route by which purine nucleotides are synthesized in DMSO-differentiated cells. The greater emphasis on synthesis of purine nucleotides by the salvage pathway has been attributed to a higher affinity of the salvage enzymes for the shared substrate PRPP than that possessed by amidophosphoribosyl transferase (Muller *et al.*, 1983). In PMNs separated from human blood, the activity of the *de novo* pathway was twice as high, yet the activities of the key regulatory enzymes of this pathway were several-fold lower, than those of the DMSO-differentiated cells. On the other hand, PMNs synthesized purine nucleotides by the salvage pathway at only 20% the rate of the DMSO-treated cells and the activities of the salvage enzymes were also significantly lower. These results suggest again that exposure of HL60 cells to DMSO results in cells which are differentiated to some extent but which still have striking metabolic differences from their blood cell counterparts emphasizing, once again, incomplete differentiation.

Due to the drastic inhibition of purine and pyrimidine nucleotide synthesis in DMSO-differentiated cells (Ahmed and Weidemann, 1984), and because of the significant role played by purine bases and purine analogues in cellular differentiation (Ishiguro and Sartorelli, 1985; Sokoloski and Sartorelli, 1987), the effects of four different nucleotide biosynthesis inhibitors were tested on differentiation and purine/pyrimidine metabolism in HL60 cells. These compounds have attracted attention as antineoplastic agents (Ahluwalia *et al.*, 1990). Of the four nucleotide biosynthesis inhibitors, acivicin (a glutamine analogue) (Lyons *et al.*, 1990; Kemp *et al.*, 1986) and mycophenolic acid (a specific inhibitor of IMP dehydrogenase and GMP synthetase) (Lucas *et al.*, 1983; Sokoloski *et al.*, 1986) were able to differentiate

HL60 cells, while alanosine (a specific inhibitor of adenylosuccinate synthetase) (Graff and Plagemann, 1976) and dipyridamole (an inhibitor of nucleoside and nucleic acid base transport) (Fitzgerald, 1987) failed to do so. Differentiation of HL60 cells by acivicin and mycophenolic acid was associated with substantial decreases in both the guanylate and adenylate pools and appeared to be dependent on the state of depletion of intracellular GTP; consistent with the observations of Lucas *et al.* (1983) and Nichol *et al.* (1989). Simultaneous addition of guanosine or guanine to mycophenolic acid-treated cells restored the GTP pool and prevented differentiation from occurring. Adenine or adenosine had no such effect, while hypoxanthine and inosine partially reversed the differentiation. These observations are consistent with those of Wright (1987) and Knight *et al.* (1987), who showed that maturation of HL60 cells by dimethylformamide, RA and tiazofurin (an inhibitor of IMP dehydrogenase) is hampered by the addition of exogenous guanine and guanosine. In acivicin-treated cells, simultaneous addition of guanine caused partial prevention of differentiation; the incomplete reversal of maturation in this case may have been due to depletion of CTP pools as a consequence of inhibition of CTP synthetase (Hindenburg *et al.*, 1985; Kemp *et al.*, 1986). Even though treatment of HL60 cells with alanosine and dipyridamole resulted in the depletion of guanylates, this effect was secondary to the depletion of adenylates and developed only upon prolonged exposure. In all the inhibitor-treated cells the activities of the key regulatory enzymes of *de novo* purine biosynthesis were affected. The inhibition of amidophosphoribosyl transferase in mycophenolic acid, acivicin and alanosine-treated cells probably occurred in response to the accumulation of IMP. Even though the measurable activity of HGPRT was enhanced in inhibitor-treated cells, the activity of the salvage pathway was inhibited in mycophenolic acid and alanosine-treated cells, possibly due to overlapping pathways of salvage and *de novo* purine nucleotide biosynthesis which ultimately proceed through IMP. The slight enhancement of salvage pathway activity seen in alanosine and dipyridamole-treated cells may have been due to a corresponding increase in the synthesis of adenylates stimulated by the depletion of ATP. Besides *de novo* purine nucleotide biosynthesis, *de novo* pyrimidine nucleotide biosynthesis was also

inhibited in inhibitor-treated cells, possibly as a consequence of ATP depletion. The inhibition of purine and pyrimidine nucleotide biosynthesis in mycophenolic acid, acivicin and alanosine-treated cells resulted in an increase in the steady-state concentration of PRPP, while in dipyridamole-treated cells it resulted in depletion of PRPP. The depletion may have been a consequence of the inhibition of ribose 5-phosphate synthesis by the non-oxidative pentose phosphate pathway because of the lack of thiamine pyrophosphate as a cofactor for transketolase.

Since purine and pyrimidine nucleotides play an important role in the synthesis of glycoprotein and sialic acid, the inhibition of these precursor molecules by mycophenolic acid and acivicin in HL60 cells leads to the inhibition of glycoprotein and sialic acid synthesis (Sokoloski and Sartorelli, 1985; Hindenburg *et al.*, 1985). Glycoproteins and sialic acid are important constituents of cellular plasma membranes; inhibition of their synthesis might cause changes in membrane macromolecules, possibly resulting in differentiation. Hence, it can be suggested that depletion of guanine ribonucleotide as a result of inhibition of early *de novo* purine nucleotide biosynthesis, or due to specific inhibition of *de novo* guanine nucleotide biosynthesis, may be an obligatory step in the initiation of differentiation in HL60 cells.

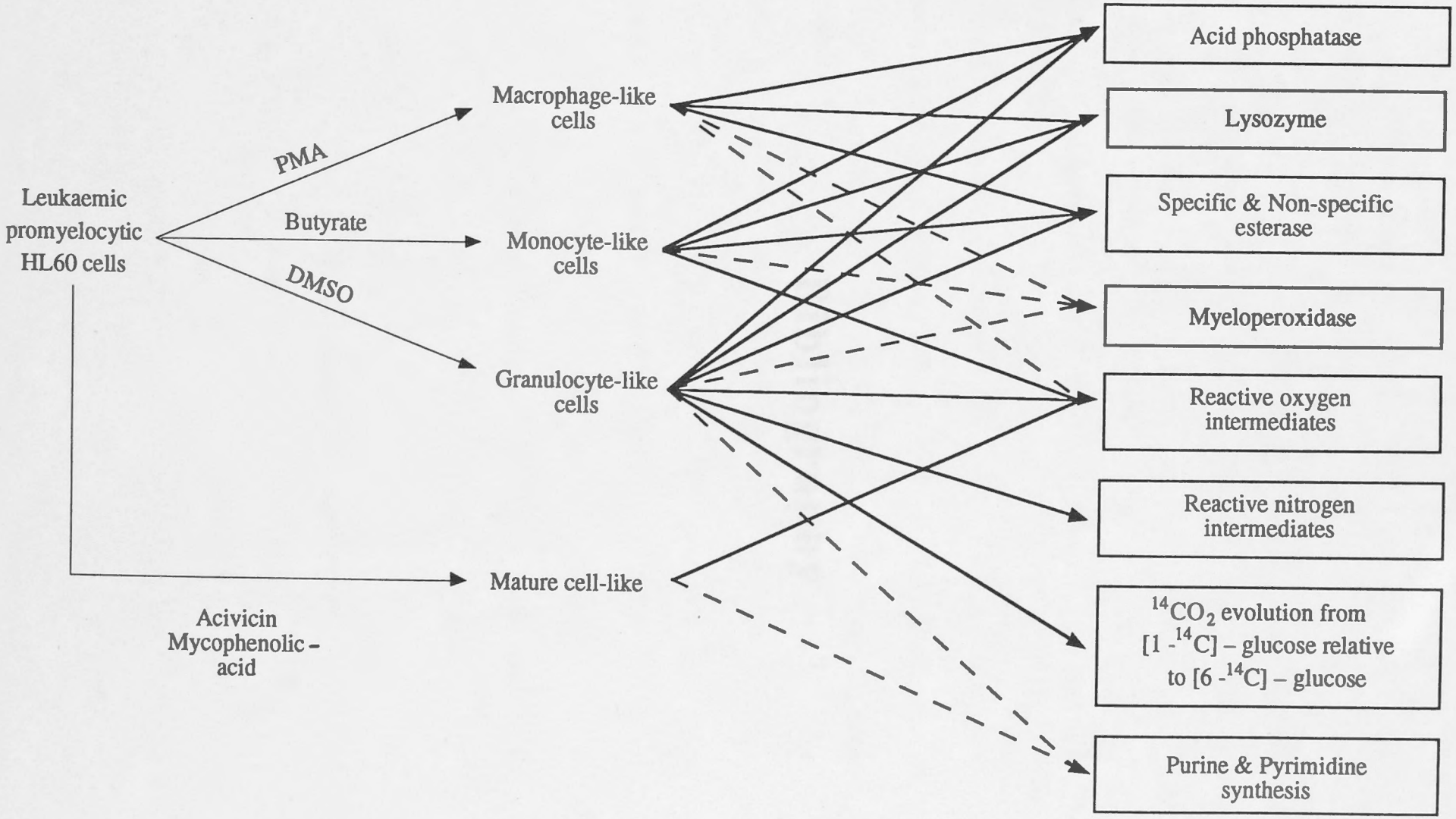
In conclusion, the present study suggests that exposure of HL60 cells to chemical agents results in their transformation to 'chemically-differentiated' cells which possess some of the functional properties of their normal cell counterparts from human blood whilst retaining striking metabolic and phenotypic differences. Thus, the induction of immature leukaemic cells to differentiation by chemical agents only partially modifies the expression of the malignant phenotype without fully expressing the characteristics of the normal phenotype, resulting in cells with 'incomplete differentiation'. Hence the end-products of HL60 differentiation induced by chemical agents possess some 'mature cell-like' properties but in other respects fail to meet several important criteria of true maturity. The differentiation of HL60 cells by chemical agents is summarized schematically in Figure 8.

Fig 8: Schematic representation of the differentiating pathways taken by HL60 cells on exposure to chemical agents.

Abbreviations used: DMSO, dimethylsulphoxide; PMA, phorbol 12-myristate 13-acetate.

Normal arrows show pathways taken by HL60 cells on exposure to the chemical agents; Dark arrows represent stimulation and dotted lines represent inhibition.

Fig 8


Leukaemic promyelocytic HL60 cells
PMA
Butyrate
DMSO
Macrophage-like cells
Monocyte-like cells
Granulocyte-like cells
Mature cell-like
Acivicin
Mycophenolic – acid
Acid phosphatase
Lysozyme
Specific & Non-specific esterase
Myeloperoxidase
Reactive oxygen intermediates
Reactive nitrogen intermediates
$^{14}CO_2$ evolution from [1 -^{14}C] – glucose relative to [6 -^{14}C] – glucose
Purine & Pyrimidine synthesis

Bibliography

Ahluwalia, G.S., Grem, J.L., Hao, Z. and Cooney, D.A. (1990) Pharmac. Ther. **46**, 243.

Ahmed, N., Williams, J.F. and Weidemann, M.J. (1991) Biochem. Int. **23**, 591.

Ahmed, N., Williams, J.F. and Weidemann, M.J. (1993) Biochem. Mol. Biol. Int. **29**, 1055.

Ahmed, N. and Weidemann, M.J. (1994) In press.

Albina, J.E., Caldwell, M.D., Williams, H.L. and Mills, C.D. (1989) J. Exp. Med. **169**, 1021.

Albina, J.E. and William, H.L. (1991) J. Surgical Res. **50**, 403.

Allen, L.M., Corrigan, M.W. and Meinking, T. (1981) Chem. Biol. Interact. **33**, 365.

Allen, R.C., Stjernholm, R.L. and Steel, R.M. (1972) Biochem. Biophys. Res. Commun. **47**, 679.

Allen, R.C. and Loose, L.D. (1976) Biochem. Biophys. Res. Commun. **69**, 245.

Anfossi, G., Gewirtz, A. and Calabretta, B. (1989) Clinical Res. **37**, 376A.

Aoki, T., Sebolt, J. and Weber, G. (1982) Biochem. Pharmac. **31**, 927.

Arch, J.R.S. and Newsholme, E.A. (1978) Essays Biochem. **14**, 82.

Ardawi, M.S.M. and Newsholme, E.A. (1982) Biochem. J. **208**, 743.

Ardawi, M.S.M. and Newsholme, E.A. (1985) Biochem. J. **231**, 713.

Ardawi, M.S.M. and Newsholme, E.A. (1983) Biochem. J. **212**, 835.

Arora, K.K. (1984) PhD Thesis, The Australian National University, ACT, Australia.

Babior, B.M. (1987) Trends Biochem. Sci. **12**, 241.

Baggiolini, M. and Wymann, M.P. (1990) Trends Biochem. Sci. **15**, 69.

Ball, E., Guyre, P., Sher, L., Glynn. J., Maliszewski, C., Baker, P. and Fanger, M. (1984) J. Clin. Invest. **73**, 1072.

Bamberger, E.G., Aggio, M.C., Lozzio, C.B. and Lozzio, B.B. (1978) Leuk. Res. **2**, 305.

Barbul, A. (1986) J. Parenteral and Enternal Nutrition **10**, 227.

Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A. and Freeman, B.A. (1990) Proc. Natl. Acad. Sci. USA. **87**, 1620.

Bergmeyer, H.U., Bernt, E., Schmidt, F. and Stork, H. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed), Academic Press, Inc. New York, London, p 1196.

Bernstein, A., Hunt, D.M., Crichley, V. and Mak, T.W. (1976) Cell **9**, 375.

Berridge, M.J. (1987) Ann. Rev. Biochem. **56**, 159.

Besterman, J.M. and Cautrecasas, P. (1984) J. Cell. Biol. **99**, 340.

Billiar, T.R., Curran, R.D., Stuehr, D.J., West, M.A., Bentz, B.G. and Simmons, R.L. (1989) J. Exp. Med. **169**, 1467.

Birnie, G.D. (1988) Br. J. Cancer **58**, 41.

Birkinshaw, J.H., Raistrick, H. and Ross, D.J. (1952) Biochem. J. **50**, 630.

Blackmore, P.F., Williams, J.F., Schofield, P.J. and Power, P.A. (1982) Int. J. Biochem. **14**, 171.

Blackmore, P.F. and Williams, J.F. (1974) Int. J. Biochem. **5**, 343.

Blough, N.V. and Zafiriou, O.C. (1985) Inorg. Chem. **24**, 3504.

Board, M., Humm, S. and Newsholme, E.A. (1990) Biochem. J. **265**, 503.

Borregaard, N., Schwartz, J.H. and Tauber, A.I. (1984) J. Clin. Invest. **74**, 455.

Boss, G.R. (1989) Proc. Natl. Acad. Sci. USA. **86**, 7174.

Boyd, A.W. and Metcalf, D. (1984) Leuk. Res. **8**, 27.

Brand, K. (1985) Biochem. J. **228**, 353.

Brand, K., Hintzenstern, J.V., Langer, K. and Fekl, W. (1987) J. Cell. Physiol. **132**, 559.

Breitman, T.R., Collins, S.J. and Kiene, B. (1980) Exp. Cell. Res. **126**, 494.

Breitman, T.R., Selonick, S.E. and Collins, S.J. (1980) Proc. Natl. Acad. Sci. USA. **77**, 2936.

Brestel, E.P. (1985) Biochem. Biophys. Res. Commun. **126**, 482.

Bretz, U. and Baggiolini, M. (1974) J. Cell Biol. **63**, 251.

Brown, G., Bunce, C.M. and Guy, G.R. (1985) Br. J. Cancer **52**, 681.

Buchannan, J.M., Smith, M.L. and Smith, R.J. (1982) Adv. Enzyme Regul. **20**, 135.

Burch, R.M. (1989) Mol. Neurobiol. **3**, 155.

Camici, M., Tozzi, M.G., Allegrini, S., Corso, A.D., Sanfilippo, O., Daidone, M.G., Marco, C.D. and Ipata, P.L. (1990) Cancer Biochem. Biophys. **11**, 201.

Cerottini, J.C. and Brunner, K.T. (1974) Adv. Immunol. **18**, 67.

Chany, C. and Cerutti, I. (1982) Int. J. Cancer **30**, 489.

Challiss, R.A.J., Lozeman, F.J., Leighton, B. and Newsholme, E.A. (1986) Biochem. J. **233**, 377.

Chen, H.W., Kandutsch, A.A. and Heiniger, H.J. (1978) Prog. Exp. Tumor Res. **22**, 275.

Chen, S.F., Cleaveland, J.S., Hollmann, A.B., Weimann, M.C., Parks, R.E. and Stoeckler, J.D. (1986) Cancer Res. **46**, 3449.

Chieu, C.P. and Lee, F. (1989) J.Immunol. **142**, 1909.

Cho-Chang, Y.S., Clair, T., Bodwin, J.S. and Hill, D.M. (1980) Biochem. Biophys. Res. Commun. **95**, 1306.

Christman, J.K. (1988) in The Status of Differentiation Therapy of Cancer, Raven Press, New York, p 47.

Christopherson, R.I. and Duggleby, R.C. (1983) Eur. J. Biochem. **134**, 331.

Christopherson, R.I. and Lyons, S.D. (1990) Med. Res. Rev. **10**, 505.

Clark, M.G., Williams, J.F. and Blackmore, P.F. (1974) Catal. Rev. **9**, 35.

Clark, M.G., Williams, J.F. and Kolos, G. (1972) J. Biochem. 3, 629.

Clark, S.C. and Kamen, R. (1987) Science **236**, 1229.

Clarke, M.F., Latallo, K.J.F., Westin, E., Smith, M. and Prochownik, E.V. (1988) Mol. Cell. Biol. **8**, 884.

Cockcroft, S. (1987) Trends Biochem. Sci. **12**, 75.

Cohen, A., Ullman, B. and Martin, D.W. (1979) J. Biol. Chem. **254**, 112.

Cohen, M.B. and Sadee, W. (1983) Cancer Res. **43**, 1587.

Cohen, L.H., Newrock, K.M. and Zweilder, A. (1979) Science **190**, 994.

Colbert, D.A., Fontana, J.A., Bode, U. and Deisseroth, A.B. (1983) Cancer Res. **43**, 229.

Coleman, P.S. and Lavietes, B.B. (1981) Crit. Rev. Biochem. **11**, 341.

Collins, S.J. (1987) Blood **70**, 1233.

Collins, S.J. and Groudine, M. (1982) Nature **298**, 679.

Collins, S.J., Gallo, R.C. and Gallagher, R.E. (1977) Nature **270**, 347.

Collins, S.J., Ruscetti, F.W., Gallagher, R.E. and Gallo, R.C. (1978) Proc. Natl. Acad. Sci. USA. **75**, 2458.

Collins, S.J., Ruscetti, F.W., Gallagher, R.E. and Gallo, R.C. (1979) J. Exp. Med. **149**, 969.

Cozzolino, F., Rubartelli, A., Aldinucci, D., Sitia, R., Torcia, M., Shaw, A. and Di Gugliclano, R. (1989) Proc. Natl. Acad. Sci. USA. **86**, 2369.

Crabtree, G.W. and Henderson, J.F. (1971) Cancer Res. **31**, 985.

Csaki, C., Szabo, C., Benyo, Z., Reivich, M. and Kovach, A.G.B. (1991) Life Sci. **49**, 1087.

Cunningham, J.M., Pattinson, J., Mehta, A.B. and Francis, G.E. (1988) Br. J. Haematol. **69**, 131.

Czok, R. and Lamprecht, W. (1974) in Methods of Enzymatic Analysis (Bregmeyer, H.U., ed), Academic Press, Inc. New York, London, p 1446.

Dacie, J.V. and Lewis, S.M. (1975) in Practical Haematology, (Livingstone, C., ed), Edinburgh, London and New York, p 32.

Dahlgren, C. and Stendahl, O. (1983) Infect. Immun. **39**, 736.

DeChatelet, L.R., Long, G.D., Shirley, P.S., Bass, D.A., Thomas, M.J., Henderson, F.W. and Cohen, M.S. (1982) J. Immunol. **129**, 1589.

Ding, A.H., Nathan, C.F. and Stuehr, D.J. (1988) J. Immunol. **141**, 2407.

Dworkin, M.B. and Dworkin-Rastl, E. (1991) Trends Biochem. Sci. **16**, 229.

Dworkin, M.B. and Dworkin-Rastl, E. (1989) Dev. Biol. **132**, 512.

Efron, D.T., Stephen, J.K., Regan, M.C., Wasserkrug, H.L. and Barbul, A. (1991) Surgery **110**, 327.

Eigenbrodt, E., Fister, P. and Reinacher, M. (1985) in Regulation of Carbohydrate Metabolism, Volume 2, (Beitner, R., ed), CRC Press, p141.

Eigenbrodt, E. and Glossman, H. (1980) Trends Pharmac. Sci. **1**, 240.

Elias, L., Wood, A., Crissman, H. and Ratliff, R. (1985) Cancer Res. **45**, 6301.

Escobedo, J.A. and Williams, L.T. (1988) Nature **335**, 85.

Faleto, D.L., Arrwo, A.S. and Macara, I.G. (1985) Cell **43**, 315.

Fantone, J.C. and Ward, P.C. (1982) Am. J. Path. **107**, 397.

Felsted, R.L., Gupta, S.K., Glover, C.J., Fischkoff, S.A. and Gallagher, R.E. (1983) Cancer Res. **43**, 2754.

Ferrero, D., Pessano, S., Pagliardi, G. and Rovera, G. (1983) Blood **61**, 171.

Feuerstein, N. and Cooper, H.L. (1984) Biochem. Biophys. Acta **781**, 239.

Fibach, E. and Sachs, L. (1974) J. Cell. Physiol. **83**, 177.

Filmus, J. and Buick, R. (1985) Cancer Res. **45**, 822.

Fisher, P.H.., Wilson, J.K.V., Risueno, C., Trump, D.L., Simon, K. and Alberti, D. (1986) Proc. Am. Ass. Cancer Res. **27**, 176.

Fischkoff, S., Brown, G. and Pollak, A. (1986) Blood **68**, 185.

Fischkoff, S., Pollak, A., Gleich, G., Testa, J., Misawa, S. and Rebu, T. (1984) J. Exp. Med. **160**, 179.

Fitzgerald, G.A. (1987) N. Engl. J. Med. **316**, 1247.

Fogh, J., Fogh, J.M. and Orfeo, T. (1977) J. Natl. Cancer Inst. **59**, 221.

Fogh, J. and Hajdu, S.I. (1975) J. Cell. Biol. **67**, 117a.

Fontana, J.A., Wright, D.G., Schiffman, E., Corcoran, B.A. and Deiseroth, A.B. (1980) Proc. Natl. Acad. Sci. USA. **77**, 3164.

Fontana, J.A., Colbert, D.A. and Deisseroth, A.B. (1981) Proc. Natl. Acad. Sci. USA. **81**, 2313.

Fontana, J.A., Reppucci, A. and Durham, J.P. (1986) Cancer Res. **46**, 2468.

Foo, D.D.Y. (1987) PhD Thesis, James Cook University of Queensland, Queensland, Australia.

Francis, G.E. and Pinsky, C.M. (1988) in The Status of Differentiation Therapy of Cancer, Raven Press, New York, p 331.

Franklin, T.J. and Cook, J.M. (1969) Biochem. J. **113**, 515.

Furchgott, R.F. (1988) in Vasodilation: Vascular Smooth Muscle, Peptides, Autonomic Nerves and Endothelium, (Vanhoutte, P.M. ed), Raven Press, New York, p 401.

Gale, G.R. and Schmidt, G.B. (1968) Biochem. Pharmac. **17**, 363.

Gale, G.R., Ostrander, W.E. and Alkins, L.M. (1968) Biochem. Pharmac. **17**, 1823.

Gallagher, R.E., Collins, S.J., Trujillo, J., McCredie, K., Aheara, M., Tsai, S., Metzgar, R., Aulakh, G., Ting, R., Ruscetti, F.W. and Gallo, R.C. (1979) Blood **45**, 713.

Garg, U.C. and Hassid, A. (1990) Biochem. Biophys. Res. Commun. **171**, 474.

Garthwaite, J., Charles, S.L. and Chesswilliams, R. (1988) Nature **336**, 385.

Gee, C., Griffin, J., Sastre, L., Miller, L., Springer, T., Peivnicciworms, H. and Roberts, T. (1986) Proc. Natl. Acad. Sci. USA. **83**, 5131.

Gilman, A.G. (1984) Cell **36**, 577.

Giovanella, B.C. and Stehlin, J.S. (1974) in Procceedings of International Workshop on Nude Mice, p 279.

Glock, G.E. McLean, P. (1953) Biochemistry **55**, 400.

Goldfarb, S. and Pitot, H.C. (1971) Cancer Res. **31**, 1879.

Gourdan, J.B. (1962) J. Embroyl. Exp. Morphol. **10**, 622.

Graff, J.C. and Plagemann, G.W. (1976) Cancer Res. **36**, 1428.

Graham, D. and Smydzuk, J. (1965) Anal. Biochem. **11**, 246.

Grant, S., Bhalla, K., Weinsten, I., Pestka, S., Milino, M. and Fisher, P. (1985) Biochem. Biophys. Res. Commun. **130**, 379.

Grassl, M. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed), Academic Press, Inc. New York, London, p 2168.

Green, L.C. Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S. and Tannenbaum, S.R. (1982) Anal. Biochem. **126**, 131.

Green, L.C., Luzuriaga, K.R., Wagner, D.A., Rand, W., Istfan, N., Young, V.R. and Tannenbaum, S.R. (1981) Proc. Natl. Acad. Sci. USA. **78**, 7764.

Greenberger, J.S. (1991) Crit. Rev. Oncol. Hematol. **11**, 65.

Grove, G.W. and Zweilder, A. (1984) Biochemistry **23**, 4436.

Gumaa, K.A. and McLean, P. (1969) Biochem. J. **115**, 1009.

Gupta, S.K., Diez, E., Heasley, L.E., Osawa, S. and Johnson, G.L. (1990) Science **249**, 662.

Gusella, J.F. and Housman, D. (1976) Cell **8**, 263.

Gutmann, I. and Wahlefeld, A.W. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed), Academic Press, Inc. New York, London, p 1464.

Halpercin, M.L., Taylor, W.M., Cheema-Dhadli, S., Morris, H.P. and Fritz, I.B. (1975) Eur. J. Biochem. **50**, 517.

Hall, T.C. and Oliverio, V.T. (1983) Med. Pediatr. Oncol. **11**, 383A.

Hammond, K.D. and Balinsky, D. (1978) Cancer Res. **38**, 1317.

Hanka, L.J. and Dietz, A. (1973) Antimicrobial Agents Chemother. **3**, 425.

Hanks, S.K., Quinn, A.M. and Hunter, T. (1988) Science **241**, 42.

Hapel, A.J., Vande Wonde, G.and Campbell, H.D. (1986) Lymphokine Res. **5**, 249.

Harker, L.A. and Kadatz, R.A. (1983) Thromb. Res. Suppl 1V, 39.

Harris, P. and Ralph, P. (1985) J. Leuk. Biol. **37**, 407.

Hassan, H.T. (1988) Haematologia **21**, 141.

Hassan, H.T. and Rees, J.K.H. (1989) Haematol. Oncol. **7**, 429.

Hassan, H.T. and Rees, J.K.H. (1989) Anticancer Res. **90**, 647.

Hawkins, R.A., Williamson, D.H. and Krebs, H.A. (1971) Biochem. J. **122**, 13.

Heinrich, P.C., Morris, H.P. and Weber, G. (1976) Cancer Res. **36**, 3189.

Hemmi, H. and Breitman, T. (1987) Blood **69**, 501.

Henderson, J.F. (1972) in Regulation of Purine Biosynthesis (ACS Monograph 170) Washington, DC, American Chemical Society, p 44.

Henderson, J.F. and Paterson, A.R.P. (1973) in Nucleotide Metabolism: An Introduction. Academic Press, New York.

Henderson J.F. and Khoo, K.Y. (1965) J. Biol. Chem. **240**, 2349.

Herberman, R.B., Nunn, M.E. and Lavrin, D.H. (1975) Int. J. Cancer **16**, 216.

Herberman, R.B. and Holden, H.T. (1978) Adv. Cancer Res. **27**, 305.

Herlyn, D. and Koprowski, H. (1982) Proc. Natl. Acad. Sci. USA. **79**, 4761.

Hershfield, M.S. and Seegmiller, J.E. (1976) J.Biol. Chem. **251**, 7348.

Hibbs, J.B., Vavrin, Z. and Taintor, R.R. (1987) J. Immunol. **138**, 550.

Hill, T.D., Dean, N.M., Mordan, L.J. Lau, A.F. and Boynton, A.L. (1990) Science **248**, 1660.

Hindenburg, A.A., Taub, R.N., Grant, S., Chang, G. and Baker, M.A. (1985) Cancer Res. **45**, 3048.

Hirohashi, S., Shimosato, Y., Nagai, K., Koide, T. and Kameya, T. (1976) Gann **67**, 431.

Holmes, E.W. (1980) Adv. Enzyme Regul. **19**, 215.

Honma, Y., Kasukabe, T. and Hozumi, M. (1978) J. Natl. Cancer Inst. **61**, 837.

Honma, Y., Kasukabe, T., Okabe, J. and Hozumi, M. (1979) Cancer Res. **39**, 3167.

Houchens, D.P., Ovejera, A.A., Sheridan, M.A., Johnson, R.K., Bogden, A.E. and Neil, G.L. (1979) Cancer Treat. Rep. **63**, 473.

Housman, D., Gusella, J., Geller, R., Levenson, R. and Weil, S. (1978) in Differentiation of Normal and Neoplastic Hematopoietic Cells, Cold Spring Harbor Conferences on Cell Proliferation, p 193.

Huang, C.K., Laramee, G.R. and Casnellie, J.E. (1988) Biochem. Biophys. Res. Commun. **151**, 794.

Hughes, S.M. (1983) FEBS Lett. **164**, 1.

Huisman, M.H., Raivio, K.O. and Becker, M.A. (1979) J. Biol. Chem. **254**, 12,595.

Hunter, D. and Cooper, A. (1985) Ann. Rev. Biochem. **54**, 894.

Hurlbert, R.B., Zimmernan, C.J. and Carrington, D.B. (1977) Proc. Am. Ass. Cancer Res. **18**, 234.

Ichikawa, Y. (1970) J. Cell. Physiol. **76**, 175.

Ignarro, L.J. (1988) in Vasodilation: Vascular Smooth Muscle, Peptides, Autonomic Nerves and Endothelium, (Vanhoutte, P.M. ed), Raven Press, New York, p 427.

Ignarro, L.J. (1990) Ann. Rev. Pharmac. Toxicol. **30**, 535.

Ihle, J.N., Keller, J., Oroszlan, S., Henderson, L.E., Copeland, T.D., Fitch, F., Prystiwsky, M.B., Goldwasser, E., Schrader, J.W., Palaszynski, E., Dy, M. and Lebel, B. (1983) J.Immunol. **131**, 282.

Imazumi, M. and Breitman, T.R. (1987) Eur. J. Haematol. **38**, 289.

Imaizumi, M., Uozumi, J. and Breitman, T.R. (1987) Cancer Res. **47**, 1434.

Imaizumi, M. and Breitman, T.R. (1988) Cancer Res. **48**, 6733.

Ishiguro, K. and Sartorelli, A.C. (1985) Cancer Res. **45**, 91.

Iyengar, R., Stuehr, D.J. and Marletta, M.A. (1987) Proc. Natl. Acad. Sci. USA. **84**, 6369.

Jackson, R.C., Morris, H.P. and Weber, G. (1977) Biochem. J. **166**, 1.

Jackson, R.C., Lui, M.S., Boritzki, T.J., Morris, H.P. and Weber, G. (1980) Cancer Res. **40**, 1286.

Johnson, G.R. (1981) in Experimental Hematolgy Today, Springer-Verlag, New York, p 13.

Johnston, R.B., Lehmeyer, J.E. and Guthrie, L.A. (1976) J. Expt. Med. **143**, 1551.

Kamoun, P.P., Schneider, E. and Dy, M. (1988) FEBS Lett. **226**, 285.

Kapuscinski, M. and Williams, J.F. (1984) Proc. Aust. Biochem. Soc. **16**, 6.

Katz, J. and Wood, H.G. (1963) J. Biol. Chem. **238**, 517.

Katzav, S., Zanca, D.M., Barbacid, M., Hedge, A.M., Isfort, R. and Ihle, J.N. (1989) Oncogene, **4**, 1129.

Kazlauskas, A., Ellis, C., Pawson, T. and Cooper, J.A. (1990) Science **247**, 1578.

Kemp, A.J., Lyons, S.D. and Christopherson, R.I. (1986) J. Biol. Chem. **261**, 14,891.

Kettle, A.J. and Winterbourn, C.C. (1988) Biochem. J. **252**, 529.

Kiessling, R., Klein, E. and Wigzell, H. (1975) Eur. J. Immunol. **5**, 112.

Kim, K.H. and Blatt, L.M. (1969) Biochemistry **18**, 3997.

Kimhi, Y., Palfrey, C., Spector, I., Burak, Y.L. and Littaner, U.Z. (1976) Proc. Natl. Acad. Sci. USA. **73**, 462.

Klabunde, R.E. (1983) Eur. J. Pharmac. **93**, 21.

Klebanoff, S.J., Vadas, M.A., Harlan, J.M., Sparks, L.H., Gamble, J.R., Agoshi, J.M. and Waltersdorth, A.M. (1986) J. Immunol. **136**, 4220.

Klebanoff, S.J. (1980) Ann. Int. Med. **93**, 480.

Knight, R.D., Mangum, J., Lucas, D.L., Cooney, D.A., Khan, E.C. and Wright, D.G. (1987) Blood **69**, 634.

Knox, W.E., Horowitz, M.L. and Friedell, G.H. (1969) Cancer Res. **29**, 669.

Knox, W.E. (1972) in Enzyme Patterns in Foetal, Adult and Neoplastic Rat Tissues, Karger, S., Basil.

Koeffler, H.P., Bar-Eli, M. and Territo, M.C. (1981) Cancer Res. **41**, 919.

Komuniecki, P.R. and Saz, H.J. (1982) J. Parasitol. **68**, 221.

Kornfeld, R. and Kornfeld, S. (1980) in The Biochemistry of Glycoproteins and Proteoglycans, Plenum Press, New York, p 1.

Kovacevic, Z. and Morris, H.P. (1972) Cancer Res. **32**, 326.

Kovacevic, Z. (1974) Cancer Res. **34**, 3403.

Kovacevic, Z. and McGivan, J.D. (1984) in Glutamine Metabolism in Mammalian Tissues (Haussinger, D. and Sies, H. ed), Springer-Verlag Berlin, Heidelberg, New York, Tokyo, p 49.

Kriz, R. (1990) in Proto-Oncogenes in Cell Development (Ciba Foundation Symposium 150) (Bock, G. and Marsh, J., eds) p 112.

Kruh, J. (1982) Mol. Cell. Biochem. **42**, 65.

Kunjara, L., Sochor, M., Adeoya, A., McLean, P. and Greenbaum, A.L. (1986) Biochem. J. **234**, 579.

Kyriazis, A.P., DiPersio, L., Michael, G.J., Pisce, A.J. and Stinnett, J.D. (1978) Cancer Res. **38**, 3186.

Lang, G. and Michal, G. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed), Academic Press, Inc. New York, London, p 1238.

Latif, Z.A., Lozzio, B.B., Wust, C.J., Krauss, S., Aggio, M.C. and Lozzio, C.B. (1980) Cancer **45**, 1326.

LeBeau, M., Lemons, R.S., Espinosa, R., Lawson, R.A., Arai, N. and Rowley, J.D. (1989) Blood **73**, 647.

Lee, C.D. (1987) PhD Thesis, University of Cincinnati, Cincinnati, USA.

Leftwich, J.A. (1987) PhD Thesis, Virginia Commonwealth University, Virginia, USA.

Leglise, M.C., Dent, G.A., Ayscue, L.H. and Ross, D.W. (1988) Blood Cells **13**, 319.

Lehrer, R.I., Ganz, T., Selsted, M.E., Babior, B.M. and Curnutte, J.T. (1988) Ann. Int. Med. **109**, 127.

Lehrer, R.I. and Ganz, T. (1990) Blood **76**, 2169.

Lepoivre, M., Fieschi, F., Coves, J., Thelander, L. and Fontecave, M. (1991) Biochem. Biophys. Res. Commun. **179**, 442.

Lepoivre, M., Chenais, B., Yapo, A., Lemaire, G., Thelanders, L. and Teau, J. (1990) J. Biol. Chem. **265**, 14,143.

Lichtenstein, A.K., Ganz, T. Selsted, M.E. and Lehrer, R.I. (1988) Cell. Immunol. **114**, 104.

Lieberman, I. (1956) J. Biol. Chem. **223**, 327.

Linevsky, J., Cohen, M.B., Hartman, K.D., Knode, M.C. and Glazer, R.I. (1985) Mol. Pharmac. **28**. 45.

Longenecker, J.P. (1980) PhD Thesis, The Australian National University, ACT, Australia.

Lotem, J. and Sachs, L. (1986) EMBO. J. **5**, 2163.

Lϕvtrup-Rein, H. and Nelson, L. (1982) Exp. Cell. Biol. **50**, 162.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randell, A.J. (1951) J. Biol. Chem. **193**, 265.

Lozzio, B.B. and Machado, E.A. (1982) in The Nude Mouse in Experimental and Clinical Research, Volume Two (Fogh, J. and Giovanella, B.C. ed), Academic Press, London, New York, Paris, Tokyo, p 521.

Lu, D.J. and Grinstein, S. (1990) J. Biol. Chem. **265**, 13721.

Lubbert, M. and Koeffler, H.P. (1988) Blood Rev. **2**, 121.

Lucas, D.L., Webster, H.K. and Wright, D.G. (1983) J. Clin. Invest. **72**, 1889.

Lui, M.S., Kizaki, H. and Weber, G. (1982) Biochem. Pharmac. **31**, 3467.

Luk, G.D., Civin, C.I., Weissman, R.M. and Baylin, S.B. (1982) Science **216**, 75.

Lund, P. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed), Academic Press, Inc. New York, London, p 357.

Lyons, S.D. (1989) PhD. Thesis, University of Sydney, Sydney, Australia.

Lyons, S.D., Sant, M.S. and Christopherson, R.I. (1990) J. Biol. Chem. **265**, 11377.

Machado, E.A., Lozzio, B.B., Lozzio, C.B., Lair, S.V. and Aggio, M.C. (1977) Cancer Res. **37**, 3995.

Machado, E.A., Gerard, D.A., Lozzio, C.B., Lozzio, B.B., Mitchell, J.R. and Golde, D.W. (1984) Blood **63**, 1015.

Maeda, M. and Ichikawa, Y. (1973) Gann **64**, 265.

Magrinat, S., Mason, N., Shami, P.J. and Weinberg, J.B. (1992) Blood **80**, 1880.

Maitra, U., Stringer, E.A. and Chaudhuri, A. (1982) Ann. Rev. Biochem. **51**, 869.

Makishima, M., Honma, Y., Hozumi, M., Sampi, K., Hattori, M., Ishikawa, I., Ogura, H., Kawahara, N., Kanniwa, T. and Motoyoshi, K. (1991) Biochem. Biophys. Acta. **1094**, 1.

Marie, J.P., Izaguirre, C.A., Civin, C.I., Mirro, J. and McCullock, E.A. (1981) Blood **58**, 708.

Markert, M., Adrews, P.C. and Babior, B.M. (1984) Meth. Enzymol. **105**, 362.

Marks, P.A. and Rifkind, R.A. (1978) Ann. Rev. Biochem. **47**, 419.

Marletta, M.A., Yoon, P.S., Iyengar, R., Leaf, C.D. and Wishnok, J.S. (1988) Biochemistry **27**, 8706.

Marletta, M.A. (1989) Trends Biochem. Sci. **14**, 488.

Martin, D.W. (1972) Anal. Biochem. **46**, 239.

McCall, B., Boughton-Smith, N.K., Palmer, R.M.J., Whittle, B.J.R. and Moncada, S. (1989) Biochem. J. **261**, 293.

McKenna, S.M. and Davies, K.J.A. (1988) Biochem. J. **254**, 685.

Medina, M.A., Sanchez, J.F., Marquez, F.J., Perez, R.J., Quesada, A.R. and Castro, N. (1988) Biochem. Int. **16**, 339.

Mehta, J.L., Lawson, D.L., Nicolini, F.A., Ross, M.H. and Player, D.W. (1991) Am. J. Physiol. **261**, H327.

Meltzer, M.S. (1976) Cell. Immunol. **22**, 176.

Mendelsohn, N., Gilbert, H.S., Christman, J.K. and Acs, G. (1980) Cancer Res.**40**, 1469.

Metcalf, D. (1984) in The Hematopoietic Colony Stimulating Factors, Elsevier Amsterdam.

Metcalf, D. (1989) Cancer Res. **49**, 2305.

Metcalf, D. (1988) in The Molecular Control of Blood Cells. Boston: Harvard University Press.

Metcalf, D., Johnson, G.R., (1979) J. Cell. Physiol. **99**, 159.

Metcalf, J.A., Gallin, J.I., Nauseef, W.M. and Root, R.K. (1986a) in Laboratory Manual of Neutrophil Function, Raven Press, New York, p 174.

Metcalf, J.A., Gallin, J.I., Nauseef, W.M. and Root, R.K. (1986b) in Laboratory Manual of Neutrophil Function, Raven Press, New York, p 12.

Metcalf, J.A., Gallin, J.I., Nauseef, W.M. and Root, R.K. (1986c) in Laboratory Manual of Neutrophil Function, Raven Press, New York, p 149.

Michal, G. and Beutler, H.O. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed), Academic Press, Inc. New York, London, p 1314.

Miller, A.D., Curran, T. and Verma, L.M. (1984) Cell **36**, 259.

Mitchell, R., Henning-Chubb, C., Huberman, E. and Verma, I. (1986) Cell **45**, 497.

Mitchell, H.H., Shonle, H.A. and Grindley, H.S. (1916) J. Biol. Chem. **24**, 461.

Mollering, H. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed), Academic Press, Inc. New York, London, p 350.

Moncada, S. and Higgs, E.A. (1991) Eur. J. Clin. Invest. **21**, 361.

Moncada, S., Radomski, M.W. and Palmer, R.M.J. (1988) Biochem. Pharmac. **37**, 2495.

Moreadith, R.W. and Lehninger, A.L. (1984) J. Biol. Chem. **259**, 6215.

Moore, M.A.S. (1991) Blood **78**, 1.

Morin, M.J. and Sartorelli, A.C. (1984) Cancer Res. **44**, 2807.

Morris, H.P. (1965) Advan. Cancer Res. **9**, 227.

Morrison, D.K., Kaplan, D.R., Escobedo, J.A., Rapp, U.R., Roberts, T.M. and Williams, L.T. (1989) Cell **58**, 649.

Muller, R. and Wagner, E.F. (1984) Nature **311**, 438.

Muller, R., Slamon, D.J., Tremblay, J.M., Cline, M.J. and Verma, I.M. (1982) Nature **299**, 640.

Muller, R., Tremblay, J.M., Adamson, E.D. and Verma, I.M. (1983) Nature **304**, 454.

Muller, R., Muller, D. and Guilbert, L. (1984) EMBO J. **3**, 1887.

Muller, M.M., Kraupp, M., Chiba, P. and Rumpold, H. (1983) Adv. Enzyme Regul. **21**, 239.

Murthy, Y.K.S., Thiemann, J.E. and Coronelli, C. (1966) Nature **211**, 1198.

Nakahata, T., Gross, A.J., Ogawa, M. (1982) J. Cell. Physiol. **113**, 455.

Nakahata, T. and Ogawa, M. (1982) Proc. Natl. Acad. Sci. USA. **79**, 3843.

Nakayasu, M., Terada, M., Tamura, G. and Sugimura, T. (1980) Cell. Biol. **77**, 409.

Nathan, C.F. and Hibbs, J.B.Jr. (1991) Curr. Opin. Immunol. **3**, 65.

Nathans, D., Lau, L.F., Christy, B., Hartzel, S., Nakabeppu, Y. and Ryder, K. (1988) Quant. Biol. **53**, 893.

Newburger, P.E., Chovaniec, M.E., Greenburger, J.S. and Cohen, H. (1979) J. Cell. Biol. **82**, 315.

Newburger, P.E., Baker, R.D., Hansen, S.L., Duncan, R.A. and Greenberger, J.S. (1982) Nature **299**, 61.

Newburger, P.E., Baker, R.D., Hansen, S.L., Duncan, R.A. and Greenberger, J.S. (1981) Cancer Res. 41, 1861.

Newrock, K.M., Cohen, L.H., Hendricks, M.B., Donelly, R.J. and Weinberg, E.S. (1976) Cell **14**, 327.

Newsholme, P., Curi, R., Gordon, S. and Newsholme, E.A. (1986) Biochem. J. **239**, 121.

Nguyen, B.T., Sayed, Y.M. and Sadee, W. (1984) Cancer Res. **44**, 2272.

Nichol, K.E., Chitneni, S.R., Moore, J.O. and Weinberg, J.B. (1989) Blood **74**, 1728.

Nishizuka, Y. (1986) Proc. Aust. Biochem. Soc. **18**, S9.

Novogrodsky, A., Dvir, A., Ravid, A., Shkolnik, T., Stenzel, K.H., Rubin, A.L. and Zaizov, R. (1983) Cancer **51**, 9.

Oberley, L.W. and Buettner, G.R. (1979) Cancer Res. **39**, 1141.

Okazaki, T., Bell, R.M. and Hannun, Y.A. (1989) J. Biol. Chem. **264**, 19,076.

Okazaki, T., Bielawska, A., Bell, R.M. and Hannun, Y.A. (1990) J. Biol. Chem. **265**, 15,823.

Olofsson, T., Gartner, I. and Olsson, I. (1980) Scand. J. Hemat. **24**, 254.

Olsson, I. and Olofsson, T. (1981) Expt. Cell. Res. **131**, 225.

Oster, W., Mertelsmann, R. and Herrmann, F. (1989) Int. J. Cell Cloning **7**, 13.

Palmer, R.M.J., Ashton, D.S. and Moncada, S. (1988) Nature **333**, 664.

Palmer, R.M.J., Rees, D.D., Ashton, D.S. and Moncada, S. (1988) Biochem. Biophys. Res. Commun. **153**, 1251.

Palmer, R.M.J. and Moncada, S. (1989) Biochem. Biophys. Res. Commun. **158**, 348.

Palmer, R.M.J., Ferrige, A.G. and Moncada, S. (1987) Nature **327**, 524.

Pantazis, P., Lazarov, S. and Papadopoulous, N. (1981) J. Cell. Biol. **90**, 396.

Pedersen, P.L. (1978) Prog. Exp. Tumor Res. **22**, 190.

Perkins, S.L., Andreotti, P.E., Sinha, S.K., Wu, M.C. and Yunis, A.A. (1984) Cancer Res. **44**, 5169.

Plagemann, P.G.W. and Woffendin, C. (1988) Biochem. Biophys. Acta **969**, 1.

Potter, G.K., Shen, R.N. and Chino, J.W. (1985) Am. J. Physiol. **114**, 360.

Prajda, N., Katunuma, N., Morris, H.P. and Weber, G. (1975) Cancer Res. **35**, 306.

Prajda, N. (1985) Adv. Enzyme Regul. **24**, 207.

Puck, T.T. (1987) Somatic Cell Mol. Genetics **13**, 451.

Pullen, G. and Hosking, C. (1985) Clin. Exp. Immunol. **62**, 304.

Racker, E. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed), Academic Press, Inc. New York, London, p 1342.

Rajkumar, T.V., Woodfin, B.M. and Rutter, W.J. (1966) Methods in Enzymology **9**, 491.

Ralph, P., Moore, M.A.S. and Nilsson, K. (1976) J. Expt. Med. **143**, 1528.

Reddy, G.P. and Pardee, A.B. (1980) Proc. Natl. Acad. Sci. USA. **77**, 3312.

Reem, G.H. and Friend, C. (1975) Proc. Natl. Acad. Sci. USA. **72**, 1630.

Reitzer, L.J., Wice, B.M. and Kennell, D. (1980) J. Biol. Chem. **255**, 5616.

Rennick, D., Jackson, J., Yang, G., Wideman, J., Lee, F. and Hudak, S. (1989) Blood, **73**, 1828.

Restrepo, D., Kozody, D.J. and Knauf, P.A. (1987) Am. J. Physiol. **253**, C619.

Reyland, M.E., Scott, R.B., Keife, W.E. and Cooper, L.W. (1986) Leuk. Res. **10**, 1183.

Rodbell, M. (1980) Nature **284**, 17.

Roitt, I. (1984) in Essential Immunology, Blackwell, Oxford.

Rosen, H. and Klebanoff, S.J. (1976) J. Clin. Invest. **58**, 50.

Rosenberg, H. (1959) J. Chromat. **2**, 487.

Rossi, F.(1986) Biochem. Biophys. Acta **853**, 65.

Rovera, G., Santoli, D. and Damsky, C. (1979) Proc. Natl. Acad. Sci. USA. **76**, 2779.

Rozengurt, E. (1986) Science **234**, 161.

Rubanyi, G.M., Ho, E.H., Cantor, E.H., Lumma, W.C. and Botelho, L.H.P. (1991) Biochem. Biophys. Res. Commun. **181**. 1392.

Rutter, W.J. and Lardy, H.A. (1958) J. Biol. Chem. **233**, 374.

Sabine, J.R. (1976) in Control Mechanisms in Cancer, Raven Press, New York.

Sabine, J.R., Abraham, S. and Morriss, H.P. (1968) Cancer Res. **28**, 46.

Sabine, J.R. (1975) Prog. Biochem. Pharmac. **10**, 269.

Sabine J.R. (1976) Eur. J. Cancer **12**, 299.

Sachs, L. (1978) Nature **274**, 535.

Sachs, L. (1987) Proc. Roy. Soc. Lond. **231**, 289.

Sahyoun, N., Besterman, W.M., Hsieh, T., Sander, M., Levine, H., Chang, K. and Cuatrecasas, P. (1986) Proc. Natl. Acad. Sci. USA. **83**, 1603.

Sant, M.E., Lyons, S.D. McClure, L.K., Szabados, E. and Christopherson, R.I. (1989) Cancer Res. **49**, 2645.

Sariban, E., Mitchell, T. and Kufe, D. (1985) Nature **316**, 64.

Sato, C., Kojima, K., Meynzawa, T., Nishizawa, K., Okayama, M. and Oguri, K. (1980) Cancer Res. **25**, 41.

Sauer, L.A., Dauchy, R.T., Nagel, W.O. and Morris, H.P. (1980) J. Biol. Chem. **255**, 3844.

Scher, W. and Friend, C. (1978) Cancer Res. **38**, 841.

Schmidt, H.H.W., Seifert, R. and Bohme, E. (1988) FEBS Lett **244**, 357.

Schlessinger, J. (1988) Trends Biochem. Sci. **13**, 443.

Schöbitz, B., Wolf, S., Christopherson, R.I. and Brand, K. (1991) Biochem. Biophys. Acta **1095**, 95.

Scholar, E.M. and Calabresi, P. (1973) Cancer Res. **33**, 94.

Schonthal, A. (1990) Cell Signall. **2**, 215.

Schrader, J.W., Berne, R.M. and Rubio, R. (1972) Am. J. Physiol. **223**, 159.

Schwartz, E.L. and Sartorelli, A.C. (1981) Cancer Res. **42**, 2651.

Scuderi, P., Nez, P.A., Duerr, M.L., Wong, B.J. and Valdez, C.M. (1991) Cell. Immunol. **135**, 299.

Segal, A.W. (1989) J. Clin. Invest. **83**, 1785.

Shellam, G.R. and Hogg, N. (1977) Int. J. Cancer **19**, 212.

Simmers, R.N., Webber, L.M., Shannon, M.F., Garson, O.M., Wong, G., Vadas, M.A. and Sutherland, G.R. (1987) Blood, **70**, 330.

Simpson, D.P. and Adam, W. (1975) J. Biol. Chem. **250**, 8148.

Singh, M., Singh, V.N., August, J.T. and Horecker, B.L. (1974) Arch. Biochem. Biophys. **165**, 240.

Skubitz, K.M., Zhen, Y. and August, J.T. (1982) Blood **59**, 586.

Slaughter, R.S. and Barnes, E.M. (1979) Arch. Biochem. Biophys. **197**, 349.

Sigma Diagnostic Procedure No 91 (1988) St Louis, USA.

Smith, P.J. (1986) Carcinogenesis **7**, 423.

Smyth, J.F., Poplack, D.G., Holiman, B.J., Leventhal, B.G. and Yarboo, G. (1978) J. Clin. Invest. **62**, 710.

Sochor, M., Kunjara, S., Greenbaum, A.L. and McLean, P. (1989) J. Develop. Physiol. **12**, 135.

Sokoloski, J.A. and Sartorelli, A.C. (1987) Cancer Res. **47**, 6283.

Sokoloski, J.A. and Sartorelli, A.C. (1986) Mol. Pharmac. **28**, 567.

Sokoloski, J.A., Blair, O.C. and Sartorelli, A.C. (1986) Cancer Res. **46**, 2314.

Solling, H. and Esmann, V. (1975) Anal. Biochem. **68**, 664.

Solomon, D.H., Raynal, M.C., Tejwani, G.A. and Cayre, Y.E. (1988) Proc. Natl. Acad. Sci. USA. **85**, 6904.

Sordat, B. and Bogenmann, E. (1980) In Immunodeficient Animals for Cancer Research (Sparrow, S., ed), Macmillan Press, New York, p 145.

Souza, L.M., Boone, T.C., Gabrilove, J.L., Lai, P.H., Zsebo, K.M., Murdock, D.C., Chazin, V.R., Bruszewski, J., Lu, H., Chen, K.K., Barendt, J., Platzer, E., Moore, M.A.S., Mertelsmann, R. and Welte, K. (1986) Science, **232**, 61.

Sparatore, B., Patrone, M., Passalacque, M., Melloni, E. and Pontremoli, S. (1991) Biochem. Biophys. Res. Commun. **179**, 153.

Srivastava, A.K. (1985) Biochem. Biophys. Res. Commun. **126**, 1042.

Stanley, E.R., Bartocci, A., Patinkin, D., Rosendaal, M. and Bradley, T.R. (1986) Cell **45**, 667.

Steigbigel, R.J., Lambert, L.M. and Remington, J.S. (1974) J. Clin. Invest. **53**, 131.

Stuehr, D.J. and Marletta, M.A. (1987) J. Immunol. **139**, 518.

Stuehr, D.J. and Marletta, M.A. (1987) Cancer Res. **47**, 5590.

Sugiura, M., Shafman, T., Mitchell, T., Griffin, J. and Kufe, D. (1984) Blood **63**, 1153.

Sweeney, M.J., Hoffman, D.H. and Esterman, M.A. (1972) Cancer Res. **32**, 1972.

Sweeney, M.J., Cline, J.C. and Williams, R.H. (1969) Proc. Am. Assoc. Cancer Res. **10**, 91.

Takahashi, S., Konishi, Y., Nakatani, K., Invi, S., Kojima, K. and Shiratori, T. (1978) J. Natl. Cancer Inst. **60**, 925.

Takeda, Y., Tominaga, T., Tei, N., Kitamura, M., Taga, S., Murase, J., Taguchi, T. and Miwatani, T. (1975) Cancer Res. **35**, 2390.

Tanaka, H., Abe, E., Mujaura, C., Kuribayashi, T., Konno, K., Nishii, Y. and Suda, T. (1982) Biochem. J. **204**, 713.

Tarella, C., Ferrero, D., Gallo, R.C., Pagliardi, G.L. and Ruscetti, F.W. (1982) Cancer Res. **42**, 445.

Terada, M., Nudel, U., Fibach, E., Rifkind, R.A. and Marks, P.A. (1978) Cancer Res. **38**, 835.

Till, J.E., McCulloch, E.A. and Siminovitch, L. (1964) Proc. Natl. Acad. Sci. USA. **51**, 29.

Todd, R., Griffin, J., Ritz, J., Nadler, L., Abrahams, T. and Schlossman, S. (1981) Leuk. Res. **5**, 491.

Trentin, J.J. (1970) in Regulation of Hematopoiesis. New York, Appleton-Century-Crofts, p 161.

Tso, J.Y., Bower, S.G. and Zalkin, H. (1980) J. Biol. Chem. **255**, 6734.

Tsuda, T., Fukumoto, Y., Hamamori, Y., Yamashita, T. and Takai, Y. (1987) J. Biochem. **102**, 1579.

Tucker, R.W., Chang, D.T. and Mendelobun, K. (1989) J. Cell. Biochem. **39**, 139.

Tyagi, A.K. and Cooney, D.A. (1983) Trends Pharmac. Sci. **4**, 299.

Tyagi, A.K. and Cooney, D.A. (1984) Adv. Pharmac. Chemther.**20**, 69.

Ullman, B., Clift, S.M., Cohen, A., Gudas, L.J., Levinson, B.B., Wormsted, M.A. and Martin, D.W. (1979) J. Cell. Physiol. **99**, 139.

Ullrich, A. and Schlessinger, J. (1990) Cell **61**, 203.

Van Dyke, K., Trush, M., Wilson, M., Stealey, P. and Milles, P. (1977) Microchem. J. **2**, 463.

Vanzant, G. and Goldwasser, E. (1977) Science **198**, 733.

Vanzant, G. and Goldwasser, E. (1979) Blood **53**, 946.

Volk, W.A. (1966) Methods Enzymol. **9**, 38.

Vorbrodt, A., Meo, P. and Rovera, G. (1979) J. Cell Biol. **83**, 300.

Wahl, M., Nishibi, S., Sah, P.G., Rhee, S.G. and Carpenter, G. (1988) Proc. Natl. Acad. Sci. USA. **86**, 1568.

Wang, J., Komarov, P., Sies, H. and Groot, H. (1991) Biochem. J. **279**, 311.

Watford, M., Lund, P. and Krebs, H.A. (1979) Biochem. J. **178**, 589.

Watt, S.M. and Visser, J.W.M. (1992) Cell Prolif. **25**, 263.

Weber, G., Queener, S.F. and Ferdinandus, J.A. (1971) Adv. Enzyme Regul. **9**, 63.

Weber, G., Jackson, R.C., Williams, J.C., Goulding, F.J. and Eberts, T.J. (1977) Adv. Enzyme Regul. **15**, 53.

Weber, G., Trevisani, A. and Heinrich, P.C. (1974) Adv. Enzyme Regul. **12**, 11.

Weber, G., Lui, M.S., Natsumeda, Y. and Faderan, M.A. (1983) Adv. Enzyme Regul. **21**, 53.

Weber, G. (1983) Cancer Res. **43**, 3466.

Weber, G., Prajda, N., Lui, M.S., Denton, J.E., Aoiki, T., Sebolt, J., Zhen, Y.S., Burt, M.E., Faderman, M.A. and Reardon, M.A. (1982) Adv. Enzyme Regul. **20**, 75.

Weber, G., Lui, M.S., Sebolt, J. and Faderan, M.A. (1984) in Glutamine Metabolisn in Mammalian Tissues (Haussinger, D. and Sies, H. ed) Springer-Verlag, Berlin Heidelberg, p 278.

Weidemann, M.J., Peskar, B.A., Wrogemann, K., Reitschel, E.Th., Staudinger, H. and Fischer, H. (1978) FEBS Lett. **89**, 136.

Weil, S.C. Rosner, G.L., Reid, M.S., Chisholm, R.L., Farber, N.M., Spitznagel, J.K. and Swanson, M.S. (1987) Proc. Natl. Acad. Sci. USA. **84**, 2057.

Weisbrod, S. (1982) Nature **297**, 289.

Weiss, L. (1980) in Cancer Campaign, Vol 4 (Grundmann, H. ed), Gustav Fischer, New York, p 53.

Williams-Ashman, H.G., Coppoc, G.L. and Weber, G. (1972) Cancer Res. **32**, 1924.

Williams, J.F. and Blackmore, P.F. (1983) Int. J. Biochem. **15**, 797.

Williams, J.F., Blackmore, P.F. and Power, P.A. (1974) I.R.C.S. Med. Sci. Libr. Compend. **2**, 132.

Williams, J.F., Clark, M.G. and Blackmore, P.A. (1978a) Biochem. J. **176**, 241.

Williams, J.F., Blackmore, P.F. and Clark, M.G. (1978b) Biochem. J. **176**, 257.

Williams, J.F., Arora, K.K. and Longenecker, J.P. (1987) Int. J. Biochem. **19**, 749.

Williams, R.H., Lively, D.H., Delong, D.C., Cline, J.C., Sweeney, M.J., Poore, G.A. and Larsen, S.H. (1968) J. Antibiotics **21**, 463.

Williamson, D.H. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed), Academic Press, New York, London, p 1679.

Winterbourn, R.C., Garcia, R.C. and Segal, A.W. (1985) Biochem. J. **228**, 583.

Wohlhueter, R.M., Marz, R. and Plagemann, P.G.W. (1978) J. Membrane Biol. **42**, 247.

Wolf, D. and Ratter, V. (1985) Proc. Natl. Acad. Sci. USA. **82**, 790.

Wong, G.G., Witek-Giannotti, J.S., Temple, P.A., Kriz, R., Ferenz, C., Hewick, R.M., Clark, S.C., Ikebuchi, K. and Ogawa, M. (1988) J.Immunol. **140**, 3040.

Wright, C.D., Mulsch, A., Busse, R. and Osswald, H. (1989) Biochem. Biophys. Res. Commun. **160**, 813.

Wright, D.G. (1987) Blood **69**, 334.

Wu, X., Shao, G., Chen, S., Wang, X. and Wang, Z. (1989) Leuk. Res. **13**, 869.

Yamada, M. and Kurahashi, K. (1984) J. Biol. Chem. **259**, 3021.

Yarden, Y. and Ullrich, A. (1988) Ann. Rev. Biochem. **57**, 443.

Yatani, A., Mattera, R., Codina, J., Graf, R., Okabe, K., Padrell, E., Iyengar, R., Brown, A.M. and Birabaumen, L. (1988) Nature **336**, 680.

Yen, A., Reece, S.L. and Albright, K. (1984) J. Cell. Physiol. **118**, 277.

Yen, A. (1985) Exp. Cell. Res. **156**, 198.

Yen, A., Freeman, L., Powers, V., Sant, R.V. and Fishbaugh, J. (1986) Exp. Cell. Res. **165**, 139.

Yokata, J., Asano, S., Ivamoto, A., Yosikura, H. and Miwa, S. (1984) Leuk. Res. **8**, 1085.

Yoneda, O., Alsina, M.M., Garcia, J.L. and Mundy, G.R. (1991) Endocrinology **129**, 683.

Young, D.C., Wagner, K. and Griffin, J.D. (1987) J. Clin. Invest. **79**, 100.

Yourno, J., Walsh, J., Kornatowski, G., O'Connor, D. and Kumar, A. (1983) Blood **63**, 238.

Zhen, Y., Lui, M.S. and Weber, G. (1983) Cancer Res. **43**, 1616.

Zielke, H.R., Ozand, P.T., Tildon, J.T., Sevdalian, D.A. and Cornblath, M. (1975) J. Cell. Physiol. **95**, 41.

Appendix
Computation Procedures for Calculation of t statistic

Each experiment was conducted three times and and differences between the control variables (HL60 cells or normal cells separated from peripheral human blood) and other experimental variables (differentiated cells) were tested for significance by undertaking the student's t-test for unpaired samples at the 10% confidence level. The unpaired sample t-tests were undertaken because it is assumed that the variance of the two means are not equal since the means of the two sample groups are independent.

The use of t-test is appropriate when the sample size is small and when it is assumed that samples are drawn from normal distribution. The following calculating procedures were followed in deriving the t statistic.

First the sum of squares about the sample mean for each sample was determined, which was labeled as SS_1 and SS_2. A combined sum of squares was formed by $SS_1 + SS_2$. The sum of squares for the first group , SS_1 has $(n_1 - 1)$ degrees of freedom and, for the second, SS_2 has $(n_2 - 1)$ degrees of freedom. The total degrees of freedom is therefore (n_1+n_2-2). The loss of two degrees of freedom occurred because two sum squares about two means were computed. The combined estimate of variance is:

$$s^2 = \frac{SS_1 + SS_2}{n_1 + n_2 - 2}t$$

The standard error of $\bar{x}_1 - \bar{x}_2$ is

$$\sqrt{\frac{s^2}{n_1} + \frac{s^2}{n_2}} = \sqrt{s^2\left\{\frac{1}{n_1} + \frac{1}{n_2}\right\}}$$

having $(n_1 + n_2 - 2)$ degrees of freedom. The hypothesis that the difference between $\bar{x}_1 - \bar{x}_2$ is zero was carried out by computing the t statistic. $\bar{x}_1$ represents mean of sample group one and $\bar{x}_2$ represents mean of sample group two.

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{s^2\,(1/n_1 + 1/n_2)}}$$ which would follow the t distribution with $(n_1 + n_2 - 2$ d.f.) if the null hypothesis is true.

Source: Bland, M. (1987), *An Introduction to Medical Statistics,* Oxford Medical Publications, pp. 172-74.