THE DEVELOPMENT OF AN IN VIVO MODEL OF DRUG-INDUCED TERMINAL DIFFERENTIATION OF LEUKAEMIC CELLS.

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SUMMARY.

The technique of growing human leukaemic cells in diffusion chambers was developed to enable chemicals to be assessed for their ability to induce terminal differentiation. Chambers were constructed using 0.45um filters and contained 150ul of serum-free HL-60 cells at a density of $1x10^6$ cells/ml. The chambers were implanted into CBA/Ca mice and spontaneous terminal differentiation of the cells to granulocytes was prevented by the use of serum-free medium. HL-60 promyelocytic leukaemia cell growth, in a lucite chamber with a Millipore filter, was optimised by use of a lateral incision site. Under these conditions there was an initial growth lag of 72 hours and a logarithmic phase of growth for 96 hours; the cell number reached a plateau after 168 hours of culture in vivo.

The amount of drug in the plasma of the animals and in chambers that had been implanted for 5 days, was determined after a single ip injection of equitoxic doses of N-methylformamide, N-ethylformamide, tetramethylurea, N-dibutylformamide, N-tetramethylbutylformamide and hexamethylenebisacetamide. Concentrations of both TMU and HMBA were obtained in the plasma and in the chamber which were pharmacologically effective for the induction of differentiation of HL-60 cells in vitro, that is 12mM TMU and 5mM HMBA. A 4 day regime of treatment of animals implanted with chambers demonstrated that TMU and HMBA induced terminal differentiation of 50% and 35%, respectively, of the implanted HL-60 cells to granulocyte-like cells, assessed by measurement of functional and biochemical markers of maturity. None of the other agents attained concentrations in the plasma that were pharmacologically effective for the induction of differentiation of the cells in vitro and were unable to induce the terminal differentiation of the cells in vivo.

Key Mords : Differentiation, diffusion chamber, in vivo HL-60.

Dedicated to My Family

We shall not cease from exploration,
And the end of all our exploring,
Will be to arrive where we started,
And know the place for the first time.

T.S. Elliot (1942).

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ABBREVIATIONS

RPMI 1640	Rosewell Park Memorial Institute 1640 Medium.
FCS	Foetal Calf Serum.
HS	Horse Serum.
MeOH	Methanol.
EtOH	Ethanol.
DMSO	Dimethylsulphoxide.
NMF	N-Methylformamide.
NEF	N-Ethylformamide.
TMU	Tetramethylurea.
DBF	N-Dibutylformamide.
TMBF	N-Tetramethylbutylformamide.
HMBA	Hexamethylene bisacetamide.
TPA	12-o-Tetradecanoylphorbol-13-acetate.
RPA	12-o-Retinoylphorbol-13-acetate.
RA	Retinoic Acid.
NBT	Nitrobluetetrazolium.
MoAb	Monoclonal Antibodies.
NSE	Non-Specific Esterase.
C'	Complement.
ip	intraperitoneal.
sc	subcutaneous.
im	intramuscular.
GLC	Gas Liquid Chromatography.
CP	Cyclophosphamide.
LD10	Lethal Dose (10%).
MTD	Maximum tolerated dose
se	Standard error.
AUC	Area under the curve

CSF	Colony stimulating factor
NCI	National Cancer Institute
MGI	Macrophage and Granulocyte inducing factor
AML	Acute Myeloid Leukaemia
CML	Chronic Myeloid Leukaemia

SECTION 1 INTRODUCTION

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1.1. The Search for New Chemotherapeutic Strategies.

A number of *in vitro* systems have been used to examine the potential of a variety of agents to induce the terminal differentiation of malignant cells. Few attempts have been made to conduct such experiments under conditions of appropriate drug pharmacokinetics *in vitro*, and fewer have utilised an *in vivo* assay of drug-induced differentiation. It is, therefore, still unclear whether drug-induced differentiation is only an *in vitro* phenomenon as it has only been tentatively suggested to occur *in vivo*. The work presented in this thesis is the development and characterisation of an *in vivo* diffusion chamber system and the study of terminal differentiation of HL-60 promyelocytic leukaemia cells under conditions of known drug pharmacokinetics to determine whether differentiation can be induced *in vivo*.

The approach to the treatment of cancer involves a

multifaceted attack utilising different treatment modalities including surgery, irradiation, chemotherapy and immunotherapy. The first option, surgery, is only applicable when the tumour is easily accessible but may present a problem in ensuring that all the tumour cells are removed during the operation. Radiotherapy has produced good remissions in some cancers but is almost as toxic to normal cells as malignant cells causing severe side-effects including myelotoxicity. In addition, radiotherapy is only successful in oxygenated tissues where free radicals can form from oxygen and the centres of many solid tumours are necrotic, poorly vascularised and hypoxic therefore containing a large number of radiation-resistant cells (Prestayko, 1984).

Chemotherapy as an effective treatment for some forms of malignant disease has gradually gained acceptance over the past 40 years. The early discovery that the nitrogen mustards could occassionally bring about regression of lymphomas and leukaemias (Gilman & Philips, 1946) was the starting point for the expansion of research into cancer chemotherapy which has led to the development of many different classes of anti-cancer chemicals. The ideal cytotoxic drug would kill cancer cells selectively, completely sparing the host tissues (McElwain, 1978). Since drugs used in cancer chemotherapy interfere with the biosynthesis of DNA, RNA and protein they are toxic to all cells both normal and malignant (Prestayko, 1984). Their

apparently selective action depends on many different factors among which are the dosage, timing and route of administration, the high rate of cell loss from many tumours (Iverson, 1967) and the capacity of some normal tissues to proliferate more rapidly than the tumours and thus repair drug-induced damage before the tumour can do so (Baserga, 1965).

Chemotherapy has, however, improved the survival of patients with a wide variety of malignancies, particularly leukaemias (Catovsky, 1984), Hodgkins' and non-Hodgkins' lymphoma (Longa & DeVita, 1984), ovarian and cervical cancer (Sausville & Young, 1984), testicular cancer (Williams & Stoter, 1984) and breast cancer (Loprinzi & Carbonne, 1984). Unfortunately, only a few responses have been achieved using chemotherapy for lung cancer (Hansen & Rorth,1984), colorectal cancer (Smith & Goldberg, 1984) and head and neak cancers (Taylor, 1984).

The use of combination chemotherapy has also frequently been proved successful for some malignancies (Frei, 1972., Alexanian et al, 1972). Effective combinations, in terms of therapeutic gain, can emerge from :

a)adequate manipulation of growth kinetic properties - based on simultaneous or sequential treatment with drugs that exert maximum lethal effects at different stages in the cell cycle. For example, vincristine arrests cells in

mitosis and bleomycin kills cells optimally in mitosis. Therefore, if vincristine was administered first to enrich the fraction of cells in mitosis, and bleomycin was given some time later when the mitotic index was increased then the effects of bleomycin would be enhanced. This schedule has been used as effective chemotherapy in the treatment of some solid tumours (Spigel & Coltman, 1974).

b)selective use of drugs that elicit less than additive toxic effects while providing at least additive therapeutic effects. Skipper (1974) has shown that animal models can be used as effective screening systems to predict potentiation of toxic effects.

c)biological synergy- such as the clinically effective synergistic pairs of vincristine and prednisolone, melphelan and prednisolone and synergistic pairs noted in experimental animal systems such as cyclophosphamide and cis-platinum, MeCCNU and CCNU (Goldin, 1973).

A fourth category exists in which miscellaneous pharmacological properties are exploited, such as the "rescue" effect elicited by citrovorum factor after methotrexate therapy (Bertino et al, 1972).

Classical anti-tumour chemotherapy generally relies upon cytodestruction as a means of removing the malignant cells from the body but this also seriously damages any rapidly

proliferating normal cells and tissues, in particular the bone marrow, causing leukopaenia, thrombocytopaenia and anaemia, the lining of the gastrointestinal tract and the basement cell membrane of the skin (Prestayko, 1984). Alopecia occurs with many chemotherapeutic regimes but is reversible on cessation of treatment. Other side-effects include allergic responses, phlebitis, neurological, pulmonary, hepatic and dermatological toxicity depending on the drug or combination administered. Long term complications include the destruction of the germ cells in the gonads, skeletal alterations and the induction of new tumours as many of the drugs used in cancer chemotherapy appear to be carcinogenic themselves (Dorr & Fritz, 1980).

New concepts for cancer chemotherapy are now required which do not involve general cytodestruction of all proliferating cells if significant advances are to be made in the treatment of the disease. One such approach would be to increase the selectivity of the drugs for the malignant cells. As early as 1904, it was proposed that antibodies might somehow serve to deliver chemically coupled toxic agents to particular tumour cells to produce a "magic bullet"(cited by Ehrlich). The aim was to improve the selectivity of cytotoxic anti-tumour drugs, targetting their anti-tumour activity by linkage to a monoclonal antibody. The immunotoxin should then kill target tumour cells and no other cells with high specificity (Collier &

Kaplan, 1984). Immunotoxic drugs have been produced by conjugating monoclonal antibodies to cytotoxic drugs including methotrexate (Garnett et al, 1983) and daunomycin (Gallego et al, 1984) which are cytotoxic to sarcoma cells expressing the antigen but not to cells of other normal or tumour tissue (Baldwin, 1984). The design of drug-antibody conjugates is still at an early stage of development and research is being directed towards the selection of appropriate cytotoxic agents and the nature of the drug-antibody linkage with respect to the use of biodegradable and non-biodegradable bonds (Baldwin, 1984). However, different monoclonals would be required for different tumours and as tumours tend to be heterogenous, not all the malignant cells might express the required antigen (Hepner, 1984). There are, therefore, still many problems to overcome before monoclonal-drug complexes may be used routinely for therapy (Davis & Rao, 1984).

It has been suggested that the development of cancer results from a breakdown in immunological surveillance (Burnett, 1970, 1972). There are, therefore, several objectives in immunotherapy including : a) to prevent or reverse the immune supression caused by other modes of cancer therapy, b)to restore immune responsiveness compromised by the disease, c) to enhance immunity to the tumour (Benjamini et al, 1983). Because chemotherapeutic agents caused such undesirable side effects, it was proposed that biological modulators, cytokines, be used in

the treatment of cancer instead of cytotoxic agents (Balkwill, 1987). The first of these, the interferons, are a family of proteins produced by somatic cells in response to a variety of stimuli, especially viral infection. There are 3 types of interferon, alpha, beta, gamma, and these are involved in antiviral and immunoregulatory activity and are able to activate NK cells so as to stimulate them to become cytotoxic and lyse a variety of target cells, including tumour cells (Ortaldo et al, 1983). The interferons have entered clinical trials against infections such as hepatitis B virus and certain respiratory viruses (Stiehm et al, 1982) and also against some leukaemias including CML and hairy cell leukaemia, and carcinomas (Balkwill, 1987). Promising results have been obtained against the viral infections but Phase II trials against gliomas, breast cancers, AML and CML have shown very few responses (Weiss et al, 1984) and it has been concluded that the alpha and beta interferons have no significant anti-tumour activity. Gamma interferon was shown to be more potent against certain tumours in experimental animals and also has more potent effects on cells in the immune system but has yet to be tested in the clinics (Fleischmann et al, 1984). A major drawback with the interferons is that they produce side-effects, although they are natural products, the most common being a flu-like syndrome including fever, loss of appetite and painful joints - suggesting that some of the symptoms of a viral infection may be due to interferons

produced by the body's defences (Quesada & Gutterman, 1983).

More recently, another cytokine, tumour necrosis factor (TNF), has been proposed for anti-tumour therapy. TNFs' are secreted by macrophages and have cytolytic/cytotoxic effects in tumour cells in culture and produce haemorrhagic necrosis in tumours in experimental animals (Fung et al, 1985., Tsjimoto et al, 1985). The TNFs' can either kill cancer cells directly or can stimulate immune cells to kill them. They also induce enhanced blood vessel permeability and fluid loss and thus can kill solid tumours by cutting off or markedly decreasing their blood supply (Balkwill, 1987). Clinical trials using TNF are currently in progress.

A third cytokine, interleukin 2 (IL-2), produced by T lymphocytes is able to stimulate the proliferation of T cells and also stimulates them to become NK cells which are then able to recognise and distroy cancer cells without damaging normal cells. IL-2 has recently entered clinical trials in patients with advanced cancer (Rosenberg et al, 1987., West et al, 1987). However, severe side effects, particularly fluid retention and cardiopulmonary stress were commonly observed and it was concluded that further attempts must be made to increase the therapeutic efficacy of this treatment and decrease its toxicity.

An alternative concept for cancer chemotherapy is the induction of the differentiation of the malignant cells.

1.2. Differentiation in Neoplasia

Neoplasia has been suggested to be a disease of aberrant cell differentiation in which the malignant cells have somehow been blocked at an immature stage but have retained the ability to proliferate continuously (Markert, 1968). He showed there were three characteristics that were important for malignancy : persistent cell division, adhesive properties of the cell membrane which would allow the cell to metastasise and specific patterns of cellular metabolism. Neoplasms were therefore produced when these various normal cell properties were abnormally combined or were in excess. Proliferation of normal stem cells with an infinite life-span is usually followed by differentiation of some of the daughter cells to a more mature phenotype which are incapable of further cell division and have a finite life-span, with a controlled balance between the differentiation and self-renewal of the stem cell (Buick & Pollak, 1984). It has been suggested that the origin and evolution of malignancy

required genetic changes that uncoupled the normal balance between proliferation and differentiation, and subsequently suggested that such malignancy was reversible (Sachs, 1985). Evidence has been obtained with various types of tumours including sarcomas (Sachs, 1978), myeloid leukaemias (Sachs, 1978) and teratocarcinomas (Stewart & Mintz, 1981) that malignant cells have not lost the genes which control normal growth and differentiation. This preservation of these normal genes was first shown in sarcomas by the finding that it was possible to reverse in cultured cells the malignant to non-malignant phenotype with a high frequency in cloned sarcoma cells (Rabinowitz & Sachs, 1968). It was suggested that normal cells contain potent tumour suppressor genes which are either missing or not expressed in malignant cells. The relationship between apparently dominant transforming oncogenes and suppressor genes has been discussed by Stanbridge (1985). Oncogenes are thought to arise from normal cellular genes (proto-oncogenes) by mutation, chromosome translocation or gene amplification which results in their continuous expression. Generally, it is thought that oncogenes have roles in the maintenance of cell proliferation in the malignant state - paralleling the functions of the proto-oncogenes in normal cell proliferation. The erb-B oncogene encodes a protein resembling a part of the EGF receptor (Downward et al, 1984). Likewise, fps, abl and src oncogenes encode protein tyrosine kinases (Hunter & Cooper, 1985) and the

product of the sis oncogene is homologous to one chain of PDGF (Doolittle et al, 1983). These oncogenes are able to transform cells which results in sustained proliferation in vitro and the ability to transform cells in vivo. However, a single activated oncogene does not appear to be sufficient for complete transformation of normal cells to malignant cells. Land et al (1983) have demonstrated that cooperation between the myc and ras oncogenes is sufficient to convert normal cells to malignant cells.

It has also been suggested that "oncogeny is blocked ontogeny" which means that cancers are caused when the cells are arrested in the foetal-embryonic stages of development (Potter et al, 1968). Blocked ontogeny would result in continued proliferation of the stem cells and daughter cells with no differentiation, which would result in a neoplasm. Interestingly many cancer cells do, in fact, possess certain characteristics of foetal-embryonic cells including isoenzymes and embryonic antigens. Alkaline phosphatase has been found to be present in carcinomas of the genital tract and stomach and is present in normal tissue in the placenta; glutaminase has been found to be present in AH-130 and 2C-18 hepatomas and in mammary carcinomas and is normally located in foetal liver (Stein et al, 1978). Carcinoembryonic antigen has been found to be associated with cell membranes in foetal and tumour tissue of the digestive tract and the antigen alpha-fetoprotein was found to be present in 60% of

hepatocarcinomas and 70% of teratocarcinomas and is produced normally by the foetal liver during development (Strong, 1980).

The reversal of malignancy is, therefore, a viable approach to the treatment of cancer as tumour cells both *in vitro* and *in vivo* have been induced to differentiate with a variety of agents as will now be described.

1.2.1 Induction of tumour cell differentiation in vitro

Differentiation has been induced in several different malignant cell lines *in vitro*. Human colon carcinoma (PLD-1) cells have been induced to differentiate to a more mature phenotype by N,N-dimethylformamide (Christensen *et al*, 1985). These cultures were examined ultrastructurally using electron microscopy and differentiation was assessed by an increased frequency of desmosomes; a direct correlation is known to exist between the number of desmosomes and degree of differentiation in some human carcinomas (Ghadially, 1980). These differentiated cells also had reduced tumorigenicity when implanted into nude mice. In contrast, the treatment of colonic adenocarcinoma cells with dimethylsulphoxide, sodium butyrate or dimethlyformamide did not reduce the tumorigenicity of these cells (Kim *et al*, 1980., Hager *et*

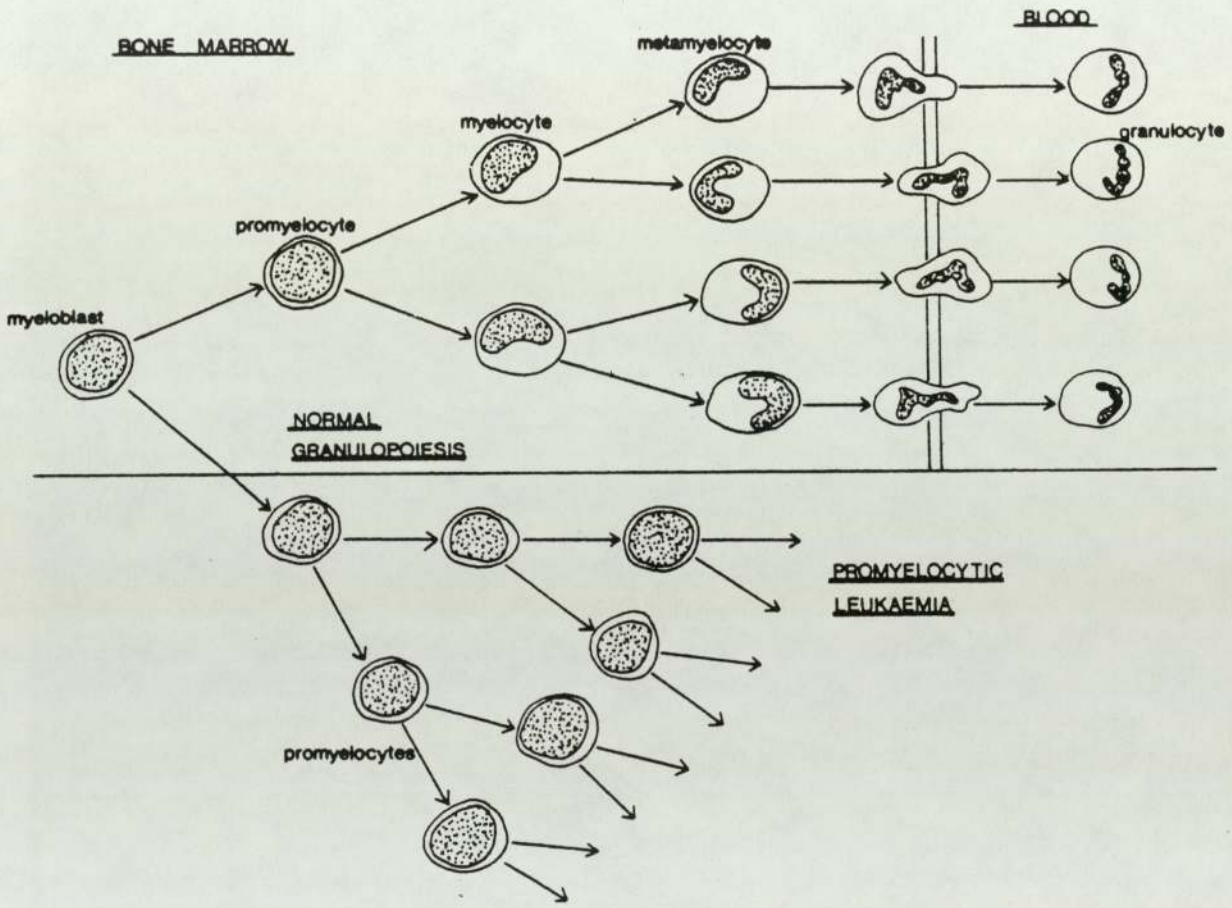
al, 1980). The cells did, however, have an increased doubling time and altered expression of certain surface antigens but these were reversible on removal of the inducer. Huberman et al (1979) have shown that human melanoma cells when treated with dimethylsulphoxide or phorbol esters were induced to differentiate as characterised by an inhibition of cell growth and melanin synthesis. Rat mammary cells have been induced to differentiate to alveolar-like cells with prostaglandin E1 or dimethylsulphoxide (Rudland et al, 1982) and these differentiated cells had reduced tumorigenicity when injected into mice.

A number of murine and human leukaemia cells have likewise been shown to differentiate to a more mature phenotype (Reviewed by Tsiftsoglou and Robinson, 1985., Sachs,1978). The HL-60 promyelocytic leukaemia cell line has been demonstrated to undergo full terminal differentiation to either granulocytes or macrophages in response to a wide range of inducers in vitro (Collins et al, 1980). See Fig. 1.1 and section 1.5..

1.2.2. Induction of differentiation in vivo

The induction of differentiation of malignant murine embryonal carcinomas to benign cystic teratomas in vivo

Fig.1.1 Diagram to show the normal differentiation pathway of granulocytes and the differentiation pathway that occurs in promyelocytic leukaemia.



has been extensively studied (Speers, 1982., Speers & Altman, 1984). In this model system the tumours were grown subcutaneously in mice and treated with either a combination of retinoic acid and DMSO or retinoic acid alone which was administered subcutaneously or intratumourly. Differentiation was assessed by the appearance of neuroepithelial and glandular structures or by a decreased tumour growth rate, decreased mitotic index, decreased extent of necrosis and increased survival time of the host animals. This work was later extended to an examination in detail of the histology of those tumours which, after differentiation induction, possessed both benign tissue and areas of mitotically active tissue (Speers & Altman, 1984). When transplanted into mice, these tumours grew and either resembled chondrosarcomas or were a glioma-chondrosarcoma mixture and were lethal. The authors suggested that <u>in vivo</u> differentiation of murine embryonal carcinomas will usually produce benign cystic teratomas but occassionally (1 per $2x10^8$ cells) will give rise to highly malignant tumours which can be predicted by examination of the primary tumour for areas of mitotically active cells. That these highly malignant, well differentiated tumours were obtained from the embryonal carcinomas was not surprising as secondary malignant changes in otherwise benign teratomas, are not unknown in humans (Scully, 1979).

In 1984, Weinberg & Muskonis demonstrated that although

differentiation of the myeloblastic leukaemia cell line (RF-AML) of the RFM/Un mouse could be induced *in vitro* with dexamethasone or TPA, this could not be repeated *in vivo* when the cells were injected ip. or iv. into the mice. It was suggested that the results of *in vitro* experiments may not be accurate predictions of the effectiveness of *in vivo* therapy. However, both the dexamethosone and TPA were dissolved in PBS prior to injection into the mice and there was no evidence to support that this was the optimum carrier system *in vivo* for the chemicals. Also, there was no mention of the distribution of the compounds *in vivo* or of the pharmacodynamics. It was possible that the TPA was degraded and made ineffective by esterases *in vivo*, or, that due to its lipophilic nature, it did not make contact with the tumour cells but was retained within peritoneal adipose tissue. Hence, these negative *in vivo* results should be viewed with caution. Similarly, Sampi *et al* (1985) reported that a discrepancy existed in the response of leukaemic cells from a relapsed patient with acute promyelocytic leukaemia to the *in vitro* and *in vivo* attempts to induce differentiation by retinoids and Actinomycin D. Some interesting findings were reported by Honma *et al* (1979) who studied the effects of differentiation inducers on the survival time of mice inoculated with sensitive and resistant M1 myeloid leukaemia cells. They showed that the sensitive M1 cells could be induced to differentiate in diffusion chambers *in*

vivo when the mice were treated with dexamethasone or lipopolysaccharide, whereas resistant M1 cells could not be induced to differentiate. Their results suggested there was a possibility of treating leukaemia in vivo with inducers of differentiation.

1.2.3. Clinical trials of Differentiating Agents

A variety of agents that will induce terminal differentiation of cell lines in vitro have been proposed as potential differentiating agents. An early report of possible in vivo differentiation of leukaemia cells was in 1975 when it was reported that a patient with acute myeloid leukaemia entered remission when treated with sodium butyrate (Leder & Leder), which is known to induce differentiation of some leukaemic cell lines in vitro (Collins et al, 1978), although no attempt was made to seek evidence of the induction of differentiation in the leukaemic cells. In 1982, 14 patients with squamous lung cell cancer were treated with a combination of a cytotoxic drug, cyclophosphamide, and a differentiating agent, dimethylsulfoxide, but no responses were observed in any of the patients (Fuks et al, 1981).

1,25 dihydroxyvitamin D_3, the active metabolite of vitamin D, is a potent inducer of terminal monocytic

differentiation of HL-60 human promyelocytic cells *in vitro* (Murao *et al*, 1983). This was administered to 18 patients with the myelodysplastic syndrome (ie. preleukaemia) as an attempt of improving haematopoiesis *in vivo* (Koeffler *et al*, 1985). Only 8 patients had a partial or peripheral blood response to the compound during its administration and no patients showed any significant improvement of peripheral blood cell or marrow blast cell counts by the end of the study. 7 patients developed leukaemia before or by the 12 weeks of study and 9 patients developed hypercalcemia.

In 1984 it was suggested that the antileukaemic effect of the drug Harringtonine on patients with acute non-lymphocytic leukaemia was via induction of differentiation with subsequent loss of proliferative capacity (Boyd & Sullivan). Harringtonine has been reported to act by inhibiting protein synthesis in a manner analogous to Actinomycin D (Stall & Knopf, 1978). On examination of the Harringtonine-treated leukaemic cells, a significant proportion of cells did show evidence of alteration to a more mature morphology, either to granulocytes or macrophages. This drug has, however, been withdrawn from clinical trials due to toxic side effects.

In 1956, N-methylformamide, which has been shown to induce differentiation of some leukaemic cells in vitro, (Collins *et al*, 1978,) was first used in clinical trials

(Myers et al, 1956) after it was found to have some activity against the sarcoma 180 in mice (Clarke et al, 1953). It was tested in 5 patients with carcinomas (epidermoid, uterine, ovarian and adenocarcinomas) and sarcomas, and showed no apparent antitumour effects whilst inducing severe hepatotoxicity which was, however, reversible once administration of the drug was halted. In contrast to many cytotoxic chemicals, NMF showed no adverse effects on the bone marrow which suggested that it might act by induction of differentiation, particularly as it is known to induce differentiation of HL-60 leukaemia cells in vitro (Collins et al, 1977, 1980). NMF was subsequently shown to be effective in combination with cyclophosphamide (Langdon & Hickman, 1985) and it has since been used in a number of clinical trials. In 1984, McVie et al reported that, in phase 1 trials, out of 15 patients with a variety of tumours including ovarian, colorectal and cervical carcinoma, only 2 partial and 2 minimal responses with nausea, vomiting and reversible hepatotoxicity as the major side effects. In a larger phase 1 trial, consisting of 35 patients, Ettinger et al (1985) reported similar side effects including considerable malaise, but again there was no myelosuppression. In a subsequent phase II trial (Eisenhauer et al, 1986) no antitumour effects were observed and the trial had to be terminated early due to severe and frequent hepatic and gastrointestinal toxicity. A major problem with the clinical trials of NMF is that

the concentration which induces differentiation *in vitro* (Collins *et al*, 1978) may either not be attainable in man or may result in a serum level which induces considerable toxicity. Alternative schedules are currently being investigated to determine whether the toxicity of NMF can be minimised.

Retinoic acid has been administered to a patient with acute promyelocytic leukaemia. After 7 weeks of daily oral treatment he went into complete remission which lasted for 6 months (Daenan *et al*, 1986). It is unclear whether the retinoic acid alone was responsible for the remission as the patient had previously been treated with cytosine arabinoside, 6-thioguanine and Adriamycin. Ara-C is an inducer of differentiation of HL-60 promyelocytic cells (Munroe *et al*, 1984), but further chemotherapy with these drugs had been considered too dangerous as the patient was suffering with Aspergillus pneumonia. The authors claimed that the retinoic acid had induced the differentiation of the leukaemic cells but there was no evidence to suggest this. Nilsson (1984) reported that a patient treated with retinoic acid was still in remission after 1 year with normal proportions of bone marrow promyelocytes, and was suffering only minimal side effects, namely slight desquamation of the skin and conjuctivitis. Retinoic acid therapy was administered to one patient who had acute promyelocytic leukaemia and residual malignant cells in his bone marrow. He had

previously been treated with multiple courses of chemotherapy (Fontana *et al*, 1986). The patients' leukaemia cells had been demonstrated to differentiate both morphologically and functionally when treated *in vitro* with retinoic acid. The patient achieved a complete remission and was maintained on retinoic acid for 1 year when he relapsed and later died with a population of leukaemia cells that were resistant to retinoic acid-induced differentiation. Although it remains unclear whether retinoic acid played a role in the remission induction, the ultimate relapse of the patient who had a population of leukaemic blasts that were no longer sensitive to the *in vitro*/*in vivo* differentiation effects of retinoic acid constitutes strong evidence in support of the retinoids playing a definitive role in the maintenance of this patients remission.

An analogue of retinoic acid , etretinate, was used in combination with Actinomycin D to treat a patient with acute promyelocytic leukaemia; this combination had been demonstrated to induce differentiation of the patients bone marrow promyelocytes *in vitro*. There was no antitumour effect of the combination of drugs and the patient died after 15 days of treatment (Sampi *et al*, 1985).

Koeffler *et al* (1985) investigated the ability of 1,25-dihydroxyvitamin D_3 to induce the terminal

differentiation of blast cells obtained from patients with acute myelogenous leukaemia. There was significant *in vitro* differentiation as assessed by morphology, phagocytosis and superoxide production.

The observation that retinoic acid can induce differentiation of human leukaemia cell lines, at concentrations that are pharmacologically obtainable in man, (Breitman *et al*, 1980) as well as some leukaemic cells from patients (Fontana *et al*, 1986) suggests its potential use in the treatment of this disease, although reports suggest that its use may be restricted to those patients with acute promyelocytic leukaemia. It appears inactive against more undifferentiated forms of myelogenous leukaemia (Breitman *et al*, 1981) and the retinoids are without apparent clinical efficacy in patients with melanoma (Meyskens *et al*, 1982) and metastatic breast cancer (Cassidy *et al*, 1982). It has been suggested that retinoic acid stimulates normal granulopoiesis *in vitro* by enhancing the responsiveness of the granulocyte-macrophage progenitors to colony-stimulating factor (Douer & Koeffler, 1982) but its *in vivo* mechanism of action remains unclear. Retinoic acid is currently being tested against a variety of other malignancies (Cheson *et al*, 1986).

Hexamethylene bisacetamide is a potent inducer of Friend erythroleukaemia and HL-60 cell differentiation *in vitro*

(Reuben et al, 1978., Collins et al, 1980., Chun et al, 1986). It was subsequently identified as being the most potent inducer for erythroid differentiation in the polar-planar compound chemical class and was demonstrated to induce greater than 99% of MEL cells to differentiate at a concentration of 5mM for 5 days (Reuben et al, 1976). Similarly, it was shown to induce 95% of HL-60 cells to differentiate after 6 days incubation at a concentration of 2mM (Collins et al, 1980). Unfortunately, in in vivo studies, which employed the NCIs' murine and xenograft screening tumours, HMBA exhibited no anti-tumour activity against any of the tumours. HMBA has recently entered clinical trials as a differentiating agent. It was tested in 20 patients with refractory solid tumours and was administered by continuous iv. infusion for 5 days with courses repeated every 4 weeks (Egorin et al, 1987) No objective tumour regressions were noted and the dose-limiting toxicity appeared to be metabolic acidosis, neurotoxicity consisting of agitation, hallucinations, confusion and alteration of consiousness, and thrombocytopaenia which were reversible once the treatment was halted. Nausea and vomiting were observed in many patients, as was bone marrow toxicity, and transient renal insufficiency was observed in 5 patients. This trial has, however, been useful in establishing the maximum tolerated dose and the pharmacokinetics. 5 urinary metabolites have so far been identified although the aetiologies of the major toxicities remain undefined. HMBA requires further

trials to determine whether it will be useful for differentiation chemotherapy. In particular, attention should be made to the scheduling of HMBA if the compound is to be used as a differentiating agent rather than as a cytotoxic agent. Continuous infusion, at a high dose, does not necessarily enable the compound to act as a differentiating agent whereas smaller repetitive dosing scheduling might be more suitable committing a certain percentage of cells to differentiate with each dose. Although the *in vivo* concentrations of HMBA obtained were comparable to the concentrations required *in vitro* for optimum differentiation, the drug levels attained were toxic to the patients. Therefore, if smaller doses were administered, thus reducing the toxicity to the patient, and the doses were administered more frequently, then a small percentage of cells might be committed to differentiate on each dose or would retain a commitment memory and would differentiate on subsequent administration of HMBA, with more tolerable side effects. In addition, as HMBA showed no activity against any solid tumour models *in vivo*, it might have been more useful to test it clinically against those tumours which it had shown *in vitro* activity.

Clinical efficacy has been claimed for Ara-C more often than perhaps any other potential differentiating agent (Cheson *et al*, 1986). However, it is still unclear whether this compound acts as a differentiating agent or

as a cytotoxic agent in patients. This appears to be a general problem associated with clinical trials of differentiating agents, as many of the trials have examined the antitumour activity of the drug rather than its differentiating effects, the markers of cell differentiation have not been incorporated into the response criteria. Also, the criteria for the distinction between the process of differentiation and selective cytotoxicity followed by proliferation of benign elements have not been established (Chun et al, 1986). Correlative laboratory studies are essential components of the proper clinical development of differentiating agents, yet they are rarely performed. In a recent review, (Cheson et al, 1986) cytogenetic data were available for only 15% and in vitro studies for only 10% of patients treated with low dose Ara-C. An additional problem with such trials is the lack of adequate pharmacokinetic data to predict the optimum dose and scheduling of differentiation agents due primarily to the lack of a suitable in vivo model of differentiation (Cheson et al, 1986). In 1985, a study was carried out to determine the pharmacokinetics of low-dose Ara-C (Spriggs et al). This study compared the drug levels obtained during a continuous infusion and an intermittent scheduling regime in patients. The intermittent scheduling provided peak levels of drug, which were probably more cytotoxic but which gave 6 hours during which time the drug levels were undetectable. In contrast, the continuous infusion of Ara-C provided a

constant low level exposure which might have allowed the drug to act as a true differentiating agent. Although the available data suggest that the response rates were greater for patients with acute non-lymphocytic leukaemia when they were continuously infused with the drug, rather than injected with a bolus every 12 hours, with both dosing regimes also causing cytotoxicity, a larger number of patients have to be included in the infusion trial before any definite conclusions can be made.

Low dose Ara-C therapy has also been administered to patients with acute non-lymphocytic leukaemia and myelodysplastic syndromes (Baccarani et al, 1983) with some success, the dose-limiting toxicity was the severe myelotoxicity frequently observed with Ara-C (Cheson et al, 1986).

1.3. Differentiation of Leukaemic Cells

1.3.1. Normal Haematopoiesis

Most of the red and white cells in the circulating blood are short-lived and need to be replaced constantly throughout life. This process of blood cell formation is termed haematopoiesis and occurs in the spleen and the bone marrow (Metcalf, 1985). It is a complex process and

derangements result in a number of important diseases which range from anaemia to leukaemia.

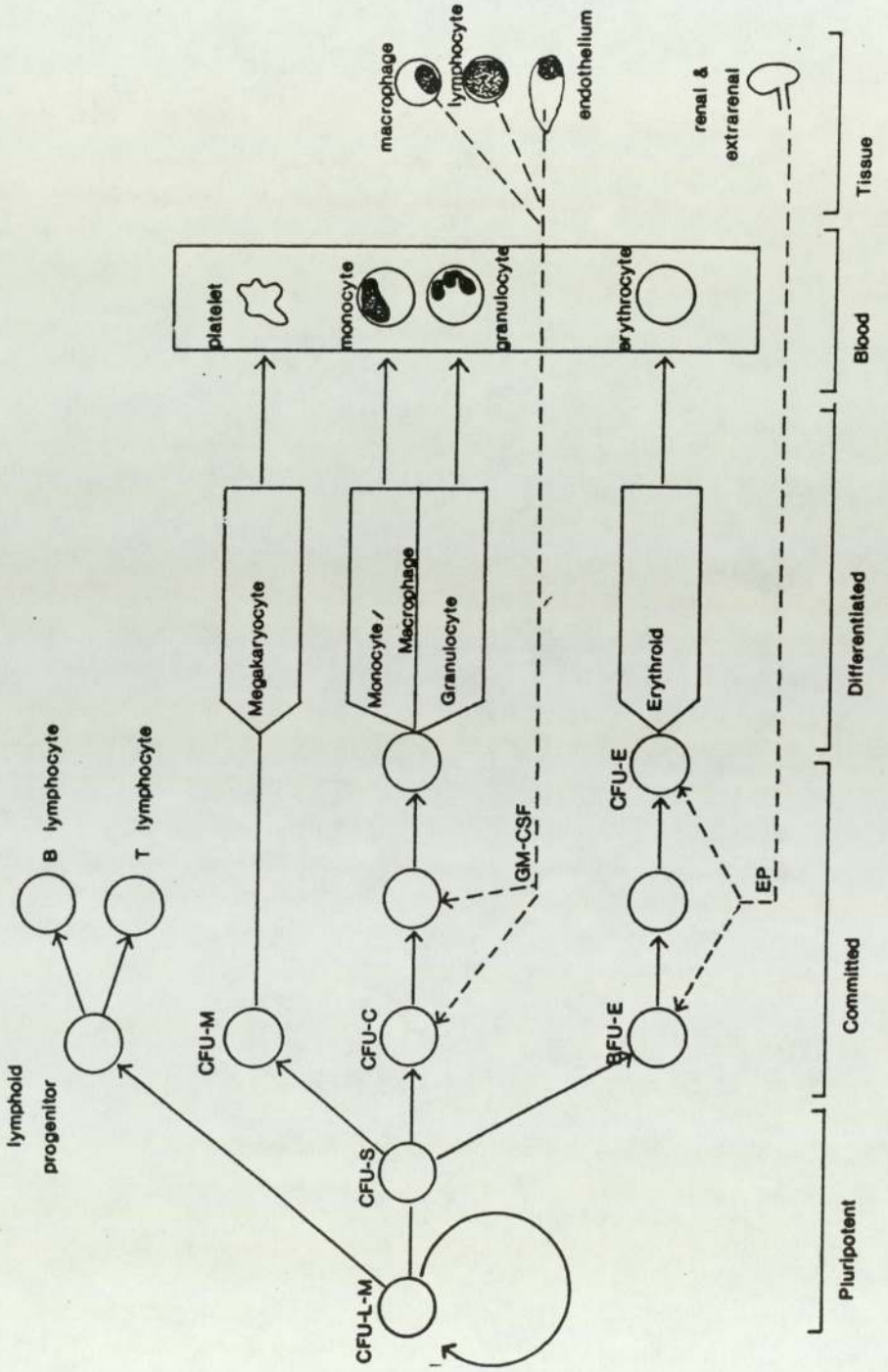
The most primitive cells in the system are the pluripotent stem cells (CSF-L-M) in the bone marrow (Metcalf, 1985) which have the potential to differentiate and give rise to 9 distinct haemtopoietic cell lineages: T & B lymphocytes, megakaryocytes (which give rise to platelets), erythrocytes, mast cells, basophils, eosinophils, neutrophils and monocyte/macrophages each with multiple maturation stages; and also possess the ability of self-renewal (Metcalf, 1985) see Fig.1.2. The progeny of these stem cells are termed CFU-S (colony-forming unit-spleen) and these cells can give rise to a range of committed stem cells : CFU-GM (granuloctye-macrophage), CFU-M (megakaryocyte) and BFU-e (burst forming unit-erythroid). All the haematopoietic cells are incapable of unstimulated cell division and this division is dependant on continuous stimulation by specific regulators (Metcalf, 1985, 1986).

The BFU-e proliferates to produce CFU-e which can then proliferate and differentiate to mature erythrocytes. This process is controlled by erythropoietin (Stohlman, 1970) which is produced primarily in the kidney in response to low circulating levels of erythrocytes, hypoxia, bleeding and irradiation (Harigaya et al, 1981). The new erythrocytes appear in the circulation 1-2 days

Fig. 1.2. Diagram to show the differentiation pathway and the growth factors involved in haematopoiesis.

(From Ruddon 1981, p90)

CFU = colony forming unit; BFU = burst forming unit; -LM = lymphoid and myeloid; -S = spleen; -GM = granulocyte and macrophage; -M = megakaryocyte; -E = erythroid; GM-CSF = granulocyte/macrophage colony stimulating factor; EP = erythropoietin.



after an increase in erythropoietin levels (Douglas, 1982)

The CFU-M cells can be induced to proliferate and differentiate to megakaryocytes which fragment to produce circulating platelets by a humoral substance thrombopoietin

The CFU-GM cells are the precursors of both granulocytic and macrophage cells and their proliferation and differentiation is controlled by a group of specific regulatory glycoproteins referred to as the CSFs' (Metcalf, 1985, 1986). Proliferation of these cells produces myeloblasts which are also capable of differentiation along the granulocytic or macrophage lineage in response to specific growth factors. Monocytes are produced from promonocytes and are located in the blood stream. They migrate or are expelled into the tissues as macrophages and these include the Kupffer cells of the liver , tissue histiocytes - which are "mobile" within certain tissues and reticulocytes - which are"fixed" within the tissues. Alternatively, the myeloblast is able to differentiate along the granulocytic lineage giving rise to promyelocytes, metamyelocytes, myelocytes and finally to mature basophils, eosinophils and neutrophilic granulocytes. The stages of granulocytic maturation can be followed by examination of the cell nucleus which changes shape from round/oval to "kidney"-shaped and finally to a segmented form in mature

granulocytes (Douglas, 1982).

An alternative pathway of haematopoiesis has been suggested by Rose et al (1985) who proposed that progenitor cells that were committed to differentiation towards megakaryocytes, erythrocytes, neutrophils, monocytes, T and B cells are arranged in a linear sequence which suggests that lineage potentials are sequentially expressed during the process of progenitor cell development. They suggested that this model could more adequately explain the co-existence of myeloid and lymphoid leukaemias and myelodysplasia in association with lymphoid and plasmacytic neoplasms than the non-linear model as previously described.

1.3.2. Myeloid Growth and Differentiation Factors

There are 4 different growth factors that have been identified as interacting to control the proliferation of granulocytes and macrophages (Metcalf, 1986). These are known collectively as the colony-stimulating factors (CSF) (Metcalf, 1985) or colony-stimulating activity (Austin et al, 1971) or MGI-1 (Lotem & Sachs, 1981).

MGI-GM or GM-CSF stimulates the proliferation and

differentiation of both granulocytes and macrophages whereas MGI-1M induces proliferation and differentiation of macrophages and MGI-1G preferentially stimulates the formation of granulocytes. The fourth class of inducer, known as multi-CSF, has the capacity to stimulate not only granulocytes and macrophages but also erythroid, eosinophil, mast, stem and multipotential cells (Metcalf, 1986). Interleukin III is a term commonly used for multi-CSF but carries with it the assumption that it is an interleukin factor exclusively of T lymphocyte origin and does not indicate the action of this molecule on erythroid and stem cell populations (Metcalf, 1985). Multi-CSF is also known by various other names including burst-promoting activity (BPA), mast cell growth factor (MCGF), haematopoietic cell growth factor (HCGF) and persisting cell-stimulating activity (Metcalf, 1986).

In addition to the CSFs, it was proposed that there are a second set of proteins which induce the differentiation of myeloid cells. These are known as differentiation-inducing factor (Olsson et al, 1984) or MGI-2 (Lotem & Sachs, 1983). The MGI-2 proteins also show heterogeneity in their molecular weights and specificity for induction of differentiation, and different types of MGI-1 may be able to induce different types of MGI-2 (Lotem & Sachs, 1983). However, it is still not clear whether there are, in fact, distinct differentiation-inducing factors. Metcalf (1986) has

claimed that the CSF's are capable of stimulating both proliferation and differentiation, whereas Lotem & Sachs (1983) have claimed that there are distinct factors which control the growth and differentiation of haemopoietic cells.

Normal myeloid precursor cells, when treated with either exogeneously added MGI-1 or compounds that are able to induce the production of MGI-1 in these cells, endogeneously produce MGI-2 thus inducing the subsequent differentiation of the myeloid cells. It is suggested that the induction of MGI-2 by MGI-1 serves as a mechanism for coupling growth and differentiation in myeloid cells, and that in certain cases , leukaemia might arise from a breakdown in this coupling (Lotem & Sachs, 1983).

The CSFs were first detected because of their unique role in stimulating haematopoiesis. However, each CSF and MGI-1 also exhibits 3 other important actions on responding cells :1) promotion of cell survival, 2) commitment to differentiation, and 3) stimulation of end cell function (Metcalf, 1985). Studies with micromanipulated paired daughter cells of G-M progenitor cells, have shown that the concentration of CSF determines the mean cell cycle time and total number of progeny produced by a single cell (Metcalf, 1980). In addition, these studies have also shown that the CSFs are able to induce irreversible commitment to one or other restricted pathway of

differentiation. Thus, high concentrations of GM-CSF induces many cells to enter the granulocytic pathway whereas low concentrations of GM-CSF permit the formation of macrophage progeny only. These growth factors also stimulate a variety of funtional activities of mature granulocytes and macrophages, for example phagocytosis of bacteria and yeasts by granulocytes and macrophages, antibody-dependent cytotoxic killing of tumour cells by granulocytes or synthesis of prostaglandin E, plasminogen activator and other regulators by macrophages (Metcalf, 1984).

1.3.3. Haematopoiesis and Leukaemia

The leukaemias are a group of diseases that arise from the stem cells of the bone marrow. The basic defect in the malignant cell type is one of faulty differentiation and uncontrolled proliferation, and results in the accumulation of abnormal cells in the marrow which eventually crowd out the normal stem cells spilling immature cell types into the peripheral blood (Ruddon, 1981). A current view of this cancerous state is that the uncontrolled proliferation is ascribable to the action of viral or cellular oncogenes whose products are either specific growth factors or receptors for such factors (Metcalf, 1986). It is, therefore, of interest to

determine whether the leukaemias are ascribable to oncogene-derived autosynthesis of CSF or CSF receptors although no sequence homology between CSFs and known oncogenes has yet been determined. It is possible that many leukaemia cells endogeously produce their own MGI-1 which maintains proliferation, but are then unable to induce the production of MGI-2 responsible for differentiation, or that the cells no longer require MGI-1 for growth but are able to continue proliferation in the absence of the growth factor due to some modification of the MGI-1 receptor (Lotem & Sachs, 1983). The HL-60 human promyelocytic cell line secretes a glycoprotein factor which stimulates HL-60 and myeloid cell growth and colony formation in vitro. This factor, known as autostimulatory activity (ASA), is distinct from CSF but has antibody cross-reactivity with CSF (Perkins et al, 1984). WEHI-3 myelomonocytic leukaemia cells have likewise been shown to produce a growth factor which is probably GM-CSF (Dexter, 1984).

Clinically, the leukaemias can be classified either according to their cell type, or as chronic or acute. Acute leukaemias generally have a rapid onset and severe impairment of cellular differentiation whereas the onset of chronic leukaemia is generally much slower and cell differentiation is not as seriously impaired (Ruddon, 1981). The more common forms of leukaemia are : acute myeloid leukaemia - characterised by the appearance of

myeloblasts or other primitive cell types in the peripheral blood; chronic myeloid leukaemia - characterised by more mature cells in the peripheral blood, these cells often carry the Philadelphia chromosome (Taussig, 1980); acute lymphocytic leukaemia - in which lymphoblast cells are the predominant cell type and chronic lymphocytic leukaemia in which more mature lymphoid cells predominate. There are also, more rarely, leukaemias of other haematopoietic cells other than white cells including erythroleukaemia (Di Guglielmos disease) and megakaryocytic leukaemias.

Various cell lines have been derived from such leukaemias which can be induced to differentiate terminally in vitro and thus serve as models with which to study the mechanisms of differentiation and drug action in such leukaemic cell populations. The K562 cell line was derived from a patient with chronic myelogenous leukaemia in blast crisis. The cells were induced to differentiate to erythroid cells which would synthesise haemaglobin when treated with hemin or butyrate (Anderson et al, 1979). These cells have also been induced to differentiate into cells of the myeloid lineage (Lozzio et al, 1981). Human histiocyte lymphoma cells, U937 cells, have been induced to differentiate to macrophage or monocytic cells when treated with phorbol ester or retinoic acid respectively, similarly KG-1 cells, which were derived from an acute myelogenous leukaemia, have been induced to differentiate

to macrophage-like cells after exposure to phorbol esters (Hemmi & Breitman, 1984). WEHI-3BD$^+$ murine myelomonocytic leukaemia cells were induced by treatment with retinoic acid to differentiate to mature granulocytes as were M1 murine myeloid leukaemia cells when treated with dexamethasone (Reiss et al, 1986). The 2 most frequently studied cell lines which will undergo differentiation in vitro are the Friend murine erythroleukaemia and HL-60 human promyelocytic leukaemia cell lines.

1.4. The Friend Murine Erythroleukaemia Cell Line

The Friend murine erythroleukaemia cell line was derived from the viral transformation of an erythroid precursor. The resulting cells appeared to be analogous to CFU-E cells ie. erythroblasts and could proliferate continuously in vitro (Friend, 1971). In response to a wide variety of inducers, including dimethylsulphoxide, hexamethylene bisacetamide, Actinomycin D, prostaglandin E1 (Collins, 1980), X-rays, irradiation, butyrate (Rifkind et al, 1984), the cells underwent terminal erythroid differentiation with a decrease in size, condensation of the nuclear chromatin, expression of erythroid antigens, and haemoglobin synthesis.

Hemin was not able to induce complete terminal differentiation of Friend erythroleukaemia cells as the cells still retained their proliferative capacity but were able to synthesise haemoglobins. The phorbol esters were able to inhibit the induction of differentiation by dimethylsulphoxide (Nomura & Oishi, 1983), as was lithium chloride (Gallicchio, 1985). Lithium was thought to act *in vivo* by increasing the circulating levels of GM-CSF, which resulted in an increased granulocytic pool and has been proposed as a means of preventing the myelosuppression caused by chemotherapy or radiotherapy (Steinbertz *et al*, 1980).

It was demonstrated in 1976 that Friend murine erythroleukaemia cells needed to be incubated with dimethylsulphoxide for a minimum of 18 hours before a significant percentage of cells were committed to differentiate (Gusella & Houseman). Two sequential programs necessary for terminal differentiation were thus proposed : the first program required the presence of inducer, and included a latent period in which irreversible commitment events did not occur whereas the second program did not require the presence of the inducer and exhibited concomitant accumulation of haemoglobin and a decrease in proliferative capacity which resulted in mature, non-proliferating, haemoglobin-expressing end cells. The individual cells became committed to the

second program in a stochastic manner and conversion of a given cell from an uncommitted to a committed state occurred with the probability determined by the genetic lineage of the cell and culture conditions. If, however, the cells were washed free of DMSO before they became committed, they retained a memory of the exposure and commitment would then proceed without a lag period if the inducer was added back to the cells (Levenson & Houseman, 1981).

1.5. The HL-60 Cell Line

The HL-60 cell line was isolated in 1977 from the peripheral blood leukocytes of an adult female with acute promyelocytic leukaemia (Collins *et al*). It was an acute human leukaemia which consisted of proliferating promyelocyte-like cells which could terminally differentiate *in vitro* in response to a wide variety of agents. These processes have been well documented, so this provided a useful model in which to study the induction of differentiation and to assess potential differentiating agents.

In response to a wide range of agents which included

retinoic acid (Breitman et al, 1980), planar-polar solvents (Spremulli & Dexter, 1984), anthracycline antibiotics (Schwartz et al, 1983) and proteolytic enzymes (Fibach et al, 1985), HL-60 cells differentiated to granulocyte-like cells with morphology, functional and biochemical markers characteristic of such cells. The cells underwent one or two divisions in the presence of the inducer before proliferation ceased and the cells differentiated. The differentiated cells had a decreased nuclear/cytoplasmic ratio, a segmented nucleus and when stimulated to produce a respiratory burst, produced the superoxide ion which was capable of reducing the dye nitrobluetetrazolium to blue/black formazan granules clearly visible by light microscopy. They also possessed the C3b complement receptor and were able to phagocytose complement-coated yeasts and gave a chemotactic response to N-formyl-methionyl-leucyl-phenylalanine (Burgess et al, 1984). A number of granulocytic cell surface markers were also induced which could be recognised using specific monoclonal antibodies (Tsiftsoglou & Robinson, 1985). From the altered nuclear morphology, it was seen that the cells progressed from promyelocytes to myelocytes and metamyelocytes and some continued further to mature banded and segmented neutrophils. Myeloperoxidase activity decreased to 20% on differentiation to either granulocytes or macrophages whereas acid phosphatase, ß-glucuronidase and lysosome activity increased on differentiation (Abita, 1984., Ross, 1985).

In contrast, when HL-60 cells were treated with the phorbol esters TPA (Abita, 1984) or RPA (Fibach et al, 1984) and 1,25 dihydroxyvitamin D_3 (Koeffler, 1985) the cells ceased proliferation and differentiated to monophage/macrophage-like cells with characteristic cell morphology. These cells developed the functional characteristics of mature end cells and were able to reduce NBT , phagocytose complement-coated yeasts, possessed monocytic non-specific esterases and adhered strongly to glass. In response to the phorbol ester TPA, however, the cells did not terminally differentiate to produce normal mature monocytes as there was a decreased level of the plasma membrane enzyme 5' nucleotidase and a decreased respiratory burst function (Newburger et al, 1981). Also, in contrast to granulocytic HL-60 differentiation, in which the cells generally had to undergo at least one cell division and the inducer had to be present for one cell cycle period (Yen et al, 1985), TPA and other monocytic inducers were able to commit the cells more rapidly to differentiation and this commitment did not require cell division (Territo & Koeffler, 1981).

Incubation of HL-60 cells with recombinant GM-CSF or murine GM-CSF induced differentiation as measured by suppression of clonogenicity and increased expression of granulocytic and macrophagic membrane markers.

Surprisingly, in contrast to other chemical inducers in which optimum differentiation was achieved after only 3/4 days incubation with the inducer, GM-CSF required 2-3 weeks to induce optimum differentiation of HL-60 cells (Begley et al, 1987).

When HL-60 cells were incubated with inducers such as DMSO or retinoic acid for 24 hours and were then washed free of the drug, only a small proportion of cells were committed and subsequently differentiated into granulocytes. However, when the cells were incubated for 48 hours with the inducer, usually 70-90% of the cells were committed to differentiate and would do so even in the subsequent absence of the inducer (Fibach et al, 1982., Yen & Albright, 1984., Yen, 1985). It was suggested therefore, that the cells had retained a memory of exposure to the inducer and that commitment events occurred in a stochastic manner as with MEL cells (Tarella et al, 1982). This commitment memory was subsequently found to be heritable through a limited number of generations and was then lost (Yen et al, 1986).

Interestingly, when HL-60 cells were incubated with the phorbol ester TPA, a small but significant percentage of cells were committed to differentiate to monocytes after only 4 hours exposure to the inducer, and 90% of cells were committed after 24 hours exposure. In contrast, after 24 hours incubation with DMSO only 10% of cells were

committed (Fibach et al, 1982). This work was subsequently extended and Fibach et al (1984) showed that the pretreatment of HL-60 cells with retinoic acid, butyric acid, DMSO or HMBA caused an enhancement of the response of the cells to the macrophage-inducing effect of TPA. The dose of TPA which was required to induce differentiation was lower and the various macrophage-specific markers appeared sooner. These results suggested that the early stages in HL-60 cell differentiation were probably common to both the granulocyte and macrophage lineages. Likewise, Yen et al (1987) concluded from their experiments using retinoic acid and 1,25 dihydroxyvitamin D_3 that commitment to differentiation was a two step process - the early events in commitment were common to both pathways and later events were involved in the determination of the different lineages.

von Melchner & Hoffken (1985) have also investigated the commitment to differentiation of HL-60 cells. In contrast to normal haemopoiesis, where committed cells continued to generate differentiated progeny and loss of self-renewal potential was invariably associated with differentiation, HL-60 cells either divided or differentiated in an exclusive manner ie. differentiation occurred as an all or non event (McCulloch, 1983). In HL-60 cells the differentiation process could be divided into two distinct components : a predeterministic

component, reflected by reversible losses of self renewal potential and a deterministic component, reflected by terminal differentiation.

The expression of cellular oncogene products was regulated in HL-60 cells during differentiation. The c-myc gene was found to be amplified between 4 and 30-fold in various sublines of HL-60 cells studied, the levels of c-myc mRNA were also increased compared with levels found in normal leukocytes and the levels were directly proportional to the gene copy number (Graham et al, 1985). Filmus & Buick (1985) have investigated the changes in c-myc expression in HL-60 cells that had been induced to differentiate with DMSO. They found that myc mRNA levels declined by day 1 of incubation with DMSO and reached a minimum after about 3 days - which paralleled the loss of clonogenicity of the cells as they differentiated. Inhibition of proliferation without differentiation was achieved by culture of the cells in reduced serum and, although c-myc mRNA levels decreased, the fall was not as dramatic as that observed during differentiation. This suggested that the inhibition of c-myc gene transcription during differentiation was not just due to inhibition of proliferation. Similar work was done by Grossot & Pitot (1985) who treated HL-60 cells, for 5 days, with either DMSO or TPA and found that transcription of c-myc gene was markedly reduced when compared with control cultures. They suggested that the decreased cellular myc mRNA levels

were due to decreased trancription of the myc proto-oncogene.

When HL-60 cells were induced to differentiate to monocytes with TPA or 1,25 dihydroxyvitamin D_3 there was an increased expression of c-fms mRNA which was preceded by decreased c-myc and increased c-fos mRNA levels (Sariban et al, 1985). It was further suggested that, as c-fms mRNA was also detectable in normal peripheral blood monocytes, this gene product may play a role in monocytic differentiation. In concordance, Sherr et al (1985) suggested that the feline c-fms proto-oncogene product was related and possibly identical to the receptor for the monocytic growth factor CSF-1.

Although the drug-induced differentiation of HL-60 cells to either granulocytes or macrophages has been well documented in vitro (see above), it is almost unknown whether this is only an in vitro phenomenon due primarily to the shortage of a suitable in vivo model of differentiation. The diffusion chamber system provides such a potential model with which to study in vivo differentiation.

1.6. Towards an In Vivo Model of Differentiation

1.6.1. History and Overview of the In Vivo Diffusion Chamber Culture System

The concept for the development of the diffusion chamber culture system dates from 1887 when Metchnikoff grew cells in a tube taken from reeds and implanted them in a host animal. Shortly thereafter, Nocard & Roux (1898) grew mycoplasma in colloidion bags which were inserted into experimental animals. In 1932, colloidion bags were again utilised to grow tissue fragments in an *in vivo* environment (Rezzesi), and this work was later extended to a study of the growth of neoplasms in similar containers implanted in the peritoneal cavity of host animals (Bisceglie, 1934). Most of these early studies were aimed at either growing cells in an *in vivo* environment where the influence of humoral factors could be investigated in the absence of cell-cell interactions, or to the growth of cells which had otherwise resisted being cultured. The desire to apply these concepts to other systems led to the development by Algire *et al* (1954) of a more sophisticated chamber consisting of a lucite ring on to which covers of filter paper membranes were attached. This type of diffusion chamber was subsequently widely used by immunologists studying the role of humoral factors in immune mechanisms (Capalbo*et al*

1964., Nettesheim et al, 1966., Benestad, 1972.)

It was recognised, at an early stage, that whilst cells grew in this system, very little was known concerning the passage of materials in or out of the chamber. Amos (1958, 1961) was the first to recognise the problems in defining the internal milieu of the chambers. He conducted a series of experiments in which he measured the rate of fluid flow into empty chambers implanted in the peritoneal cavity of mice as well as the rate of diffusion of antibodies into the chamber. Unfortunately, this early work has not been continued with detailed analysis of the kinetics of material passage into the chamber or of the composition of the internal milieu.

The first application of the diffusion chamber system to studies of the growth of bone marrow was in 1960 (Berman & Kaplan). They observed that mouse bone marrow cells which were placed intraperitoneally in diffusion chambers could survive for periods of as long as 3 weeks. It was subsequently shown that murine and rabbit lymphoid cells proliferated in the diffusion chambers (Capalbo et al, 1964) and that the culture of mouse spleen cells in diffusion chambers produced proliferating granulocytic, lymphoid cells and macrophages (Capalbo & Makinodan, 1964). The first culture of human cells in diffusion chambers implanted into rats was demonstrated in 1969 (Johnson et al). These studies involved the treatment of

peripheral blood lymphocytes with phytohemagglutinin *in vitro* followed by culture in diffusion chambers in the rat peritoneum. A marked blastogenesis similar to that found *in vitro* occurred.

The diffusion chamber technique has also been applied to the study of animal and human tumours and to the effects of irradiation or drugs on these tissues. Human cancers have been studied by heterotransplantation into animals using the diffusion chamber system (Evgenjeva, 1970., Meck *et al*, 1976). The growth kinetics of haematopoietic cells from both peripheral blood and bone marrow have been studied in patients with various blood diseases such as chronic myelocytic leukaemia (Chikkappa *et al*, 1973) or myelofibrosis with myeloid metaplasia (Ohl *et al*, 1976). In addition to adult haematopoiesis, the growth of foetal haematopoietic cells has been studied in detail through use of the diffusion chamber system (Symann *et al*, 1976).

1.6.2. Diffusion Chamber Methodology

The most common form of diffusion chamber now in use consists of a plastic ring, 13-17mm outer diameter, 2mm thick, which contains a filling hole. The important characteristics of this ring material were that it would

withstand "sterilisation" at 70-80°C without deformation and would be non-toxic to the cells which were implanted within it.

a)Membrane Filters

There are at present 3 types of filters available :

1)Acropar - a fibrous filter available with and without nylon support fibres as an inherent part of the filter,

2)Millipore - a fibrous cellulosic type composed of mixed esters of cellulose with a thickness of 150μm, and,

3)Nucleopore - a filter composed of a polycarbonate film through which are etched pores of varying sizes (Carsten, 1975).

All 3 types of wall material have proved satisfactory for growth of haematopoietic cells but recovery and growth of nucleated human blood cells and mouse bone marrow cells was found to be more efficient in the Nucleopore chamber. This is possibly because the Nucleopore material is thinner and contains "straight through" holes, as opposed to the fibrous nature of Acropar and Millipore filters. This thinner material enhances the passage of fluid

materials but may also enhance cell-cell interactions between host tissues and diffusion chamber content (Carsten, 1975)

Millipore filter membranes are available with pore sizes ranging from 0.1-0.8μm. Walker et al (1984) examined various sized filters using electron microscopy 9 days after culture to see if these membranes excluded contact between cytoplasmic processes of host cells and implanted cells. He showed that rabbit bone marrow cells would not grow in diffusion chambers that were prevented from establishing close contact with host membranes. No penetration of membranes with 0.1 or 0.22μm pore size was observed, and only shallow penetration of 0.45μm was observed. Intact cells were able to transverse the 0.8μm filters. Cell growth was comparable in the chambers constructed from 0.1, 0.22 and 0.45μm membranes. Other workers have also reported that the pore size of the membrane filter affected cell growth, Benestad & Reikvam, (1975) showed that human bone marrow cells would grow to a higher density when cultured in diffusion chambers constructed from 0.22μm filters rather than in diffusion chambers constructed from 0.1 or 0.8μm filters.

b)Chamber Assembly

The fabrication of chambers is achieved either by heat sealing the membrane filters to the ring, which requires

special equipment, or by gluing the filter to the ring using Millipore or Tensol cements. Benestad & Reikvam (1975) have shown that both types of cement were toxic to human bone marrow cells but not to tumour cells. Page et al (1986) conducted a series of diffusion chamber experiments in which the internal surface of Millipore filters were coated with Millipore cement - either in a stripe or over the entire surface of the membrane, and the diffusion chamber was filled with murine bone marrow cells. They found that cell growth was observed only on areas of the filter which were not covered with cement. They concluded both that the cement does not have a trophic effect on cell distribution and that it was toxic to the cells. Overspill of the cement onto the membrane filter also acts as a physical barrier reducing the diffusion area and transport of nutrients across the chamber walls (Carsten, 1984).

c) Implantation of Chambers

The chamber recipients normally used are rats, mice and guinea pigs with the chambers implanted intraperitoneally in rats and mice, and subcutaneously in guinea pigs (Ross & Lillywhite, 1965). Benestad & Reikvam (1975) have demonstrated that higher cell yields were retrieved from diffusion chambers carried by young, small mice rather than older mice - the results were not influenced by sex differences or the number of implants, whether single or

double, within an individual host. Further studies using the diffusion chamber technique have been performed in other animals and have included the evaluation of calf skin collagen fibres following their subcutaneous implantation within a diffusion chamber into a normal calf (Shoshman et al, 1974). In 1975, Laissue et al irradiated a goat before implanting a 4 chamber system containing autologous bone marrow cells to study the effect of irradiation on cell growth.

In 1961, diffusion chambers which contained autologous or homologous leukocytes were implanted subcutaneously into volunteer subjects (Petrakis et al). The chambers were implanted in small incisions along the axillary line of the chest. The chambers were left for periods ranging up to 6 weeks after which they were sectioned and examined microscopically . In a similar study, normal bone marrow in diffusion chambers was implanted subcutaneously both in the donor and in the identical twin brother of a subject with acute myeloblastic leukaemia (Essers et al, 1974). In a more ambitious study in 1977, (Greenberger et al), 49 patients were implanted with chambers containing autologous bone marrow, autologous and homologous lymphocytes and autologous tumour tissue. In these studies, a lucite frame measuring 1 x 14 x 25mm and containing 2 Millipore diffusion chambers was implanted subcutaneously through the skin in an area just superior to the posterior lateral aspects of the iliac crest. The

chambers' contents were evaluated at time periods ranging from 6-57 days. In general, it was found that the bone marrow did not survive as such but was replaced by fibroblasts and macrophages. The various tumours tested did not survive at all. The homologous and autologous peripheral blood leukocyte fractions underwent transformation into fibroblasts and mononuclear macrophages. Survival of homografts was proven by the use of sex chromatin marker techniques.

d)Harvesting of Diffusion Chambers

The most efficient evaluation of the cell number was by dissolution of the plasma clot within the chamber using enzymes such as pronase (Benestad, 1970)). After removal of the chamber from the host animals , the chambers were wiped free of adherent host material and incubated in vials which contained 0.025% pronase for 20-60 minutes. The chamber could then be quantitatively emptied by piercing of the chamber wall with a micropipette. Benestad & Reikvam (1975) have reported that the addition of 5% Ficoll to the pronase solution greatly increased the yield of viable CFU-C.

1.6.3. Types of Diffusion Chamber

There are generally considered to be 2 types of culture which can be propagated in diffusion chambers . In one, the cultured cells are enmeshed in a semi-solid matrix, usually consisting of a fibrin or plasma clot, or soft agar. Cell growth in these cultures occurs in clonal form. The other diffusion chamber technique is often referred to as the suspension method.

In 1974, the *in vitro* agar technique was combined with the conventional diffusion chamber technique to comprise the agar diffusion chamber method (Gordon, 1974., Gordon *et al*, 1975). In this system, one of the filter walls was replaced with a glass coverslip, allowing visualisation of colony growth, similar to that in the *in vitro* agar technique. The cells were primarily mixed with an agar medium and injected into the diffusion chamber, it was then possible to evaluate subsequent colony growth. This technique has been applied to studies of colony-forming cells (CFU-GM) in chronic myeloid leukaemia (Nilsson & Olofsson, 1985), colony formation by normal haematopoietic precursor cells (Gordon *et al*, 1975) and for the study of anthracycline antibiotic-induced leukaemic cell differentiation (Schwartz *et al*, 1983).

A plasma clot diffusion chamber was developed in 1976 (Steinberg *et al*). This allowed the assessment of

erythrocytic and granulocytic colony formation in chambers containing normal rat bone marrow implanted into untreated rats and those pretreated with endotoxin or phenylhydrazine. In the conventional diffusion chamber system, the cell suspensions were placed in diffusion chambers and, through natural processes in the host animal, a gelatin - like clot was formed. In the plasma clot diffusion chamber system, a clot was induced to form within 2-5 minutes by the addition of clotting factors to the chambers which already contained a cell suspension. The most commonly used clotting factor was 15-20% citrated bovine serum within the chamber (Steinberg et al, 1976., Niskanen & Wyandt, 1985). The chamber contents were evaluated either by harvesting the chamber clot intact (Gerard et al, 1978) or by a combination of protease clot dissolution and centrifugal force (Hsu & Trupin, 1978). Other applications of the plasma clot diffusion chamber system have included the study of murine colony-forming cells in irradiated mice (Niskanen & Wyandt, 1985), evaluating the role of erythropoietin in hypoxic conditions on the differentiation of BFU-e (Harigaya et al, 1981) and evaluating the effect of endotoxin on normal human bone marrow growth (Preister et al, 1978).

The other technique of diffusion chamber culture, as described by Algire et al, (1954) was often referred to as the suspension method. Cells were instilled into the diffusion chamber in liquid suspension and plasma clots

form within a few days *in vivo* through natural processes in the host animal. On removal, the plasma clot was lysed by enzymic incubation and the chamber contents assessed in suspension form. By use of this method it was possible to harvest virtually all viable cells from the chamber and obtain a single cell suspension (Benestad, 1970, Brevik & Benestad, 1972) It was suggested that the suspension diffusion chamber method was analogous to *in vitro* liquid suspension cultures of haemopoietic cells (Page *et al*, 1986) .

1.6.4. Multiple Diffusion Chamber Methodology

Originally, when the modern diffusion chamber technique was introduced in 1954 by Algire *et al*, it was common practice to implant one diffusion chamber per mouse. It was later demonstrated that a 25-30g mouse could comfortably accommodate the intraperitoneal implantation of two 13mm diffusion chambers and that a 200g rat could easily accommodate four 13mm diffusion chambers (Boyum *et al*, 1972), these results were confirmed in 1975 (Benestad & Reikvam). A Teflon holder was later designed which could contain 4 chambers held in place without the need for toxic glues (Gerard *et al*, 1978). This allowed for duplicate culture of two cell types within an individual

(Quesenberry et al, 1978) or serial removal of one diffusion chamber with the continued culture of the remaining chambers (Benestad et al, 1978).

An alternative methodology of multiple diffusion chamber culturing was described by Bab et al (1986). They cemented the diffusion chambers together edge to edge and implanted four diffusion chambers per 25g mouse, each pair of diffusion chambers were implanted either side of the abdominal midline to study the kinetics of differentiation of bone marrow stromal cells in vivo. All the chambers were removed simultaneously and the contents processed for histology, enzyme histochemistry, histomorphometry, biochemical analysis and alkaline phosphatase activity.

An interesting variation was the use of double or triple - set diffusion chambers which have made possible the further evaluation of humoral effects on cells grown in diffusion chambers. These consisted of two or three diffusion chambers sharing a common centre filter and allowed the evaluation of the effects of diffusable humoral factors produced in one chamber on cells cultured in the neighbouring chamber. Triple-set diffusion chambers have been utilised by Jovcic et al (1986), the middle chamber contained granulopoiesis-stimulating factor or colony-stimulating factor and the side chambers contained mouse bone marrow cells. The side chambers were assayed to assess the different stimulating action of

granulopoiesis- or colony- stimulating factor on granulopoiesis.

Other applications of multiple diffusion chambers have included the study of the inhibition of murine haematopoiesis by leukaemic cells (Quesenberry et al, 1978) and the study of diffusable factors from spleen cells (Pfeffer & Boyum, 1977).

1.6.5. Advantages and Disadvantages of the Diffusion Chamber System

There are a number of advantages and disadvantages of this culture system compared with other available culture methods. The ability to grow cells with some ease, which would not flourish in other avaiable culture systems remains as a strong advantage for the use of this system such as bone marrow stromal cells (Bab et al, 1986), Shay chloroleukaemia cells (Vilpo, 1973), haematopoietic cells (Niskanen,1985., Capalbo & Makinodan, 1964), leukaemic cells (Lotem & Sachs,1979., Ganser et al, 1985), lymphosarcoma cells (Kaplan, 1976) and multiple myeloma cell cultures (Jobin et al, 1974). It was also possible to observe the different patterns of differentiation which arose from the culture of normal bone marrow cells (Ganser

& Hoezler, 1984) and leukaemia bone marrow (Steele et al, 1977). The ability to work with an in vivo system for the analysis of humoral effects without cell-cell interaction was of importance. Lotem & Sachs (1983) have been able to study the effect of the hosts response to inflammation and foreign antibodies on the growth and differentiation of mouse myeloid leukaemia cells and HL-60 cells without the immunological problems that cell-cell interaction might involve. Also the activation and/or metabolism of compounds and the effects of their toxicity on the host may be studied, for example, cyclophosphamide. This drug per se is not cytotoxic, it is metabolised in the liver eventually to form phosphoramide mustard which is thought to be responsible for the antitumour activity of cyclophosphamide (Jarman et al, 1976).

In addition to measuring the toxic or cytogenetic effects of such compounds, the suppression of cell differentiation or other sub-lethal effects may be quantified (Carsten, 1984). In particular, the exposure of human cells to mutagens or chemicals such as methyl methanesulphonate (Brewan et al, 1970) or irradiation (Boyum et al, 1972) may be conveniently assessed in this in vivo environment without danger to the human donor.

The use of the diffusion chamber is not without some disadvantages, a major factor being the relative cost of materials, time and host animals (Benestad & Reikvam,

1975). In terms of the diffusion chamber itself, characterisation of the diffusion chambers' internal milieu has not been adequately carried out (Carsten, 1984). The growth of cells within the diffusion chamber at times shows a lack of reproducibility (Benestad, 1970).The variability of cell yields between replicate chambers can be great and the variability for ^{3}H thymidine incorporation into the cultures has been shown to be even greater (Benestad, 1970). Although the diffusion chamber system provides a closed system eliminating cell-cell interactions, this aspect may be a disadvantage when studying the growth and differentiation of haematopoietic cells from the stem cell up to the death of the differentiated daughter cell. In the bone marrow in an *in vivo* situation, the pluripotent stem cells replicate and pass through various phases of differentiation in the bone marrow before migrating out into the circulation (Dexter, 1984). Continued differentiation, aging and death take place, for the most part, in areas other than the bone marrow. This situation is not true for the diffusion chamber system where all aspects of birth, growth and death take place in a limited environment. Finally, the need for drug treatment or irradiation of host animals for stimulating certain cell growth renders this system a modified *in vivo* and not a true *in vivo* system (Ohl *et al*, 1984).

1.6.6. Applications of the Diffusion Chamber Culture System

a) Mutagenesis Studies

It is known that a number of agents require metabolic activation before being effective such as cyclophosphamide (Jarman *et al*, 1979) and by using the diffusion chamber system it has been possible to study the *in vivo* effects of the mutagen or its metabolic products directly. In 1970, the diffusion chamber system was first applied to the evaluation of the mutagenic effects of some chemicals, in particular methyl methanesulphonate (Brewan *et al*, 1970). This system has also been used to study the ability of chemicals to induce chromosome aberrations (Furukawa & Huang, 1978), and in 1984, Huang & Sirianni studied the effects of a series of known promutagens and carcinogens on 10 human lymphoid cell lines and on Chinese hamster V79 cells implanted in diffusion chambers *in vivo*.

b) Neonatal Tissue Studies

The growth and differentiation of foetal haematopoietic cells has been studied extensively using diffusion chambers *in vivo* (Symann *et al*, 1976., Weinberg *et al*, 1977). In 1980, Niskanen *et al* conducted a major

comparative study of myeloid and erythroid colonies from human foetal liver grown both *in vivo* and *in vitro*. They showed that the in vivo diffusion chamber system provided a propitious milieu for both erythroid and myeloid colony formation from human foetal liver and that there was a dominence of erythroid colonies during the earlier phase of foetal development.

c)*Normal Organ Culture*

Cells from a number of normal tissues have been successfully cultured in diffusion chambers *in vivo* (Carsten, 1984). Considerable interest has been focused on the cultivation of pancreatic cells for the elucidation of the role of these cells in obesity and diabetes (Buschard , 1975). The growth and differentiation of human cervical, vaginal and uterine epithelia has also been briefly examined (Kurtz, 1966., Tileva, 1968). Much work has concentrated on culturing the skeletal tissues where the majority of the haematopoietic progenitor cells reside and studies have ranged from examining the osteogenic potency of irradiated bone marrow implanted into diffusion chambers (Kuralesova, 1968), to measuring the effect of particle size on osteogenic activity (Shapoff *et al*, 1980).

d)Haematopoietic Cell Kinetics

Kinetic studies on haematopoiesis in the intact animal have been severely hampered by 2 main problems. Firstly, the haematopoietic tissues are open systems with continuous cell exit to and entry from the blood stream, it is therefore difficult to keep account of cell numbers (Benestad, 1972). The humoral regulation of granulopoiesis is then very difficult to study since the agents tested will often induce granulocytic export from storage pools into haematopoietic tissues (Boggs, 1967). Secondly, in kinetic analysis radioautographic techniques are often employed, and the precise diagnosis of cell types may be difficult when several cell renewal systems co-exist in the same organ (LoBue, 1970). Therefore, a segregated population with only 1/2 cell lines, where cells can grow and differentiate normally would be an ideal system for the analysis of cell kinetics (Benestad, 1972). *In vitro* cell culturing would be a convenient procedure but it has been difficult to maintain normal growth in such cultures (Woodliff, 1964). Even in the agar diffusion chamber system, haematopoietic cell growth is not optimal so that stem cells rapidly disappear from the cultures (McCulloch & Till, 1971., Metcalf & Moore, 1971). The suspension diffusion chamber technique has been demonstrated to support successfully growth of haematopoietic stem cells (Benestad, 1970., Boyum and Bergstrom, 1970), and allow the survival and proliferation

of the stem cells (Brevik et al, 1971) thus permitting kinetic analysis.

The kinetics of proliferation and differentiation have been extensively studied by Benestad (1970) who demonstrated that bone marrow cells, when cultured in diffusion chambers in mice, gave rise mainly to granulocytes and macrophages with the lymphocytic population being lost. The eosinophilic granulocytes were demonstrated to have a long life span in the chambers whereas segmented neutrophils were rapidly eliminated after a life span of approximately 2 days. Chikkappa et al (1980) showed that normal human granulocytic precursors continued growth and differentiation in the diffusion chamber at the same rate as they did in vivo whereas the granulocytes obtained from a patient with chronic myelocytic leukamia differentiated at a much increased rate in diffusion chambers than normal granulocytes.

e) Evaluation of humoral regulators

The diffusion chamber system has perhaps had its widest application for evaluating humoral inhibitors or stimulators of haematopoietic cell growth (Beran,1975, Broxmeyer, 1978). An important application of the diffusion chamber system to the understanding of haematopoietic regulation was the in vivo investigation of

the role of colony stimulating factor (Ganser & Hoezler, 1984., Lovhaug, 1978., Cronkite et al, 1979) and its inhibition as demonstrated by the reduction in diffusion chamber granulopoiesis by anti-colony-stimulating factor serum (Shadduck et al, 1978).

The plasma clot diffusion chamber has been utilized as the primary in vivo model for studying the effects of erythropoietin, the humoral regulator of erythropoiesis, (Cronkite et al, 1980). The stimulation of diffusion chamber erythropoiesis was shown to be induced by hypoxia, bleeding, polycythemia and irradiation in the host animal. (Boyum et al,1972., Harigaya et al, 1981). Exogeneously added erythropoietin, administered via a novel minipump - diffusion chamber combination implanted within a rat peritoneum, has also been demonstrated to stimulate erythropoiesis within the diffusion chamber (Carsten, 1984).

f)Immunology-Related Studies

The primary stimulus for the development of the modern diffusion chamber system (Algire et al, 1954) was for the purpose of studying humoral factors involved in the immune response. Initially the morphological and functional characteristics of lymphocytes during antibody production were studied (Capalbo et al, 1964). These

studies were later extended to an examination of the induction of the primary immune response in spleen cells (Rossi & Zaalberg, 1971), to a study of the myeloma cell immunoglobulin expression (Rohrer et al, 1977) and to other general questions as related to immune mechanisms in mammals (Jacobsen et al, 1980., Thierfelder et al, 1980).

g)Tumour cell culture

The diffusion chamber system has been used to evaluate the efficacy of tumour therapy (Gordon & Blackett, 1976., Ohl et al, 1984., Sampi et al, 1985) and has been widely utilised in tumour biology (Carsten, 1984). Ovarian cancers (Evergen'Eva 1970., Benczur, 1980), breast tumours (Shamaev et al, 1981) and human melanomas (Sviatukhin & Malenkova, 1974) have all been successfully and reproducibly cultured in diffusion chambers. Some human tumour cells have been continuously cultured in diffusion chambers for periods of several months such as malignant pleural effusion cells derived from patients with Hodgkins' disease (Boecker et al, 1975) and lymphosarcoma cells (Kaplan, 1976) - these cultures have eventually given rise to large quantities of monoclonal tumour cells available for detailed biochemical analyses and monitoring of their chromosome evolution in vivo (Boecker et al, 1984). Ehrlich ascites tumour cells have been widely studied in diffusion chambers in vivo (Brennerman & Rigby

1968), and have been suggested as a model for testing new chemotherapeutic agents *in vivo* (Ohl *et al*, 1984).

1.6.7. Differentiation using the in vivo Diffusion Chamber System

The diffusion chamber system has been utilised to study the differentiation of both human and mouse leukaemia cells *in vivo* (Gordon & Blackett, 1976. Lotem & Sachs, 1983, 1985.).

Lotem and Sachs (1978) have conducted a series of experiments utilising the diffusion chamber technique in suspension form which used a range of mouse myeloid leukaemia cells which differed genetically in their competance to be induced to undergo normal differentiation *in vitro* by macrophage and granulocyte inducing protein, MGI. They showed that leukaemia clones that could be induced to differentiate *in vitro* and even those clones that could not be induced to differentiate *in vitro* by exogeneously added MGI, could be induced to differentiate partially in diffusion chambers *in vivo*. No mature end cells were detected but the leukaemic cells were shown to possess receptors for complement and immunoglobulin Fc

suggestive of a more mature phenotype. This *in vivo* differentiation was completely inhibited using mice that had been pretreated with cyclophosphamide before chamber implantation. Their results suggested that the different leukaemia clones respond to different concentrations of MGI, which were higher in the *in vivo* model than in the *in vitro* system, and that this *in vivo* differentiation might also be regulated by cells involved in the immune response. Further experiments were thus performed using both the murine leukaemia clones and HL-60 cells in SJL/J mice. It was subsequently demonstrated that *in vivo* differentiation was regulated, to some extent, by inflammation and compounds that induce inflammation *in vivo* such as sodium caseinate, thioglycollate and bacterial lipopolysaccharide (Lotem & Sachs, 1983, 1985), or by foreign antigens such as bovine serum albumin and chicken ovalbumin (Lotem & Sachs, 1985), the presence of a xenogeneic but not a syngeneic or allogeneic, or no serum present in the chamber (Lotem & Sachs, 1983). This response required the presence of T lymphocytes as *in vivo* differentiation was inhibited in congenitally athymic nude or neonatally thymectomised mice (Lotem & Sachs, 1983).

Recent experiments have shown that the differentiation of mouse myeloid leukaemia cells in diffusion chambers *in vivo* is regulated by a cell-mediated immune response involving antigen-specific helper T lymphocytes and that this is associated with the ability of helper T cells to

produce myeloid differentiation-inducing protein, MGI-2 (Lotem & Sachs, 1986).

Anderson et al (1983) have likewise utilised the suspension diffusion chamber technique for assessment of the effect of various doses and schedules of cyclophosphamide on the growth of L1210 murine leukaemia cells in BDF_2 mice. They demonstrated that the effect of cyclophosphamide on the number of cells within the diffusion chambers was dose-related, quantifiable, reproducible and predictive of survival in leukaemia-bearing animals treated similarly. The suspension diffusion chamber technique has also been used by Hoezler et al (1977) to determine if the peripheral blood of 21 patients with different forms of acute leukaemia would differentiate in vivo. They used cells from patients with acute myeloid leukaemia, acute erythroleukaemia, acute myelomonocytic leukaemia, acute undifferentiated leukaemia and acute lymphoid leukaemia and implanted 2 diffusion chambers into irradiated CBA mice. Their results showed that many granulocytic cells were produced from diffusion chamber culture of the leukaemic blood cells and cytogenetic analysis of the cultured cells showed that many were probably descendants of the leukaemic cells as there were many clonal numerical and structural chromosome aberrations in the granulocytic cells. However, a few mitoses with normal karyotype were observed which might have been descendants from a

remaining normal stem cell population. They concluded that the leukaemic cells had differentiated to a more mature granulocytic cell during culture within the diffusion chamber due to some stimulation provided in the diffusion chamber system. Irradiation of the host animal does result in increased granulopoiesis within a diffusion chamber system (Boyum *et al*, 1972) and the effect is probably dose-dependent (Squires, 1975) but the factors which promote such growth are as yet unidentified.

The plasma clot diffusion chamber system has only rarely been used for the study of differentiation of tumours *in vivo*. Slee *et al* (1985) conducted a comparative study using solid tumours obtained from patients to determine whether this technique supported more efficient tumour growth than an *in vitro* soft-agar colony-forming assay. They showed that the morphology of the colonies produced in the plasma clot assay was superior to the morphology in the soft-agar assay, but that there was more variability in the plasma clot diffusion chamber than the soft-agar assay - possibly as a consequence of the use of animals. This work has not yet been continued with drug testing in the plasma clot system.

In contrast, experiments to measure differentiation which used the agar diffusion chamber system did not appear to have been so successful. This technique was utilised by Schwartz *et al* (1983) to study the *in vivo*

differentiation of HL-60 cells in response to marcellomycin and other anthracycline antibiotics. In these experiments, the chambers were implanted into CD-1 mice and a single dose of the drug was administered intraperitoneally. 24 hours later, the chambers were removed and transferred to the peritoneal cavity of a drug-free mouse that had received 600 rads. of whole body irradiation 4 hours previously to promote the cloning efficiency of the cells. 8 days later, the chambers were removed and the cloning efficiency and the percentage of cells capable of reducing nitrobluetetrazolium determined. The HL-60 cells were subsequently shown to have differentiated to granulocytes in response to certain of the drugs. These results which suggested the in vivo differentiation of HL-60 cells should, however, be treated with caution. The chamber contained a xenogeneic serum, FCS, shown to be antigenic and induce the in vivo differentiation of mouse myeloid leukaemia cells (Lotem & Sachs, 1983). There was no mention of the pharmacokinetics of the drugs ie. if they gained access to the chamber, and if so, for how long. Finally. as the cells were exposed to the drugs whilst in the agar chamber it is possible that the drug, if it entered the chamber, persisted in the agar for a long period of time.

Similar work was done by Selby & Steel (1982) who utilised the agar diffusion chamber to expose both human melanoma xenografts in immune-deprived mice and human

melanoma cell suspensions in diffusion chambers, to various cytotoxic drugs. It was concluded that the differences in drug exposure and cellular chemosensitivity between the chambers and the xenografts might be due to better drug penetration and retention in the chamber since their earlier work had shown relatively poor penetration by melphelan into the centres of sc. growing xenografts (Selby et al, 1979) and they suggested much caution in the interpretation of drug testing in this system.

Finally, the agar diffusion chamber technique has been utilised by Gordon & Blackett (1976) to measure the sensitivities of both murine and human haematopoietic colony-forming cells to cytotoxic drugs in vivo. Their work showed that there were significant differences between the sensitivities of murine and human bone marrow cells to cytotoxic drugs and that any extrapolation from mouse to humans should be viewed with caution. They also concluded that the presence of agar in the diffusion chamber substantially altered the response of cells to drugs, perhaps by altering drug access and/or clearance, and that the suspension diffusion chamber technique would be a more suitable model for studying the in vivo effects of drugs on human cells.

1.7. AIMS

The aims of the work presented in this thesis were threefold. Firstly, to characterise the growth of HL-60 promyelocytic leukaemia cells in diffusion chambers implanted within mice; to determine the optimum growth conditions with respect to cell number, chamber implantation site and filter pore size which would provide reproducible growth with a low percentage of spontaneous differentiation. An important feature of the model was that it would provide log phase growth conditions for 3/4 days to allow a 4 day dosing regime to be carried out. As mentioned in section 1.2.3., it might be possible to expose the cells to the inducers for short but repetitive time periods and induce differentiation *in vivo* without having to maintain a high concentration of the inducer for prolonged time periods. In addition, it was demonstrated that HL-60 cells would only differentiate *in vitro* when they were able to undergo cell replication at least once, ie when they were in log phase growth conditions (Langdon *et al*, 1986) Secondly, to determine whether or not potential differentiating agents could enter the diffusion chamber and to determine the pharmacokinetics of such agents. Thirdly, to assess the potential of this model as an *in vivo* screening system for possible differentiating agents and to facilitate the selection of suitable compounds for trial as differentiating agents in this system.

SECTION 2 MATERIALS

SECTION 2

MATERIALS

2.1 SOURCES

2.1.1 Purchased from BDH Chemicals Ltd.,Poole,Dorset.

acetone

disodium hydrogen orthophosphate

ethanol

ethoxyethanol

ß mercaptoethanol

methanol

potassium dihydrogen orthophosphate

sodium carbonate

sodium chloride

trypan blue(Gurr)

xam mountant

2.1.2 Purchased from Fisons Scientific Equipment,Loughborough, Leicester.

dimethylsulphoxide

formaldehyde solution (40%)

glacial acetic acid

hydrochloric acid

potassium chloride

sodium hydroxide

2.1.3 Purchased from Sigma Chemical Company Ltd., Poole, Dorset.

alpha-naphthyl acetate

bovine serum albumin

colchicine

ethidium bromide

ethylene glycol monomethyl ether

giemsa stain,crystalline

glycerol

hexamethylene bisacetamide

iodoacetamide

methyl green

nitrobluetetrazolium

pararosaniline

phorbol 12-myristate 13-acetate

phorbol 12-retinoate 13-acetate

poly-l-lysine

sodium azide

sodium nitrite

toluidene blue

2.1.4 OTHER PURCHASES

Aldrich Chemical Company Ltd., Gillingham, Dorset.

N-methylformamide

1,1,3,3,tetramethylurea

xylene

Difco Laboratories, Surrey.

noble agar

Fluka Chemicals Ltd., Glossop, Derbyshire.

N-dibutylformamide

N-ethylformamide

1,1,3,3 tetramethylbutylformamide

Gibco Ltd., Paisley, Scotland.

cryotubes

foetal calf serum

horse serum

25 ml tissue culture flasks

250 ml tissue culture flasks

RPMI-1640 tissue culture medium

Janssen Pharmaceutical Ltd., Grove, Oxford.

Hypnorm - fentanyl citrate 0.315mg/ml.
fluanisone 10mg/ml.

R.A.Lamb, London.

Harris haematoxylin
Navy eosin

Lagap Pharmaceutical Ltd., Guildford, Surrey.

Diazepam 10mg/ml.

Macarthys Surgical Ltd., Redditch, Warks.

black suture silk
Michel clamps

Marshall and Howlett, Kent.

Klingerflon spray

Millipore (UK) Ltd., Harrow, Middlesex.

Lucite rings (13mm diameter) filter membranes
MF cement no.1

Ortho Diagnostic Systems Ltd., High Wycombe, Bucks.

OKM1 monoclonal antibody

FITC-conjugated antibody

Oxoid, Basingstoke, Hants.

complement fixation buffer tablets

phosphate buffered saline tablets

Bantin and Kingman, Hull.

CBA/CA mice (female) 18-22g

BDF1 mice (female) 18-22g

All animals were maintained in an animal house for at least one week to ac limatise. They were fed on water and Rat and Mouse breeding diet ad libitum.

2.1.5 GIFTS

Human plasma was kindly provided by the Blood Transfusion Centre, University of Birmingham

Procine rubine MX-B was a gift from ICI Organics Division, Cheshire

M718 macrophage monoclonal antibody was kindly provided by Dako

2.2 SOLUTIONS

2.2.1 Complement fixation buffer

Oxoid CFB tablets 5
distilled water to 500ml

This gave a solution equivalent to 0.575g barbitone, 0.185g soluble barbitone, 8.5g sodium chloride, 0.168g magnesium chloride, 0.028g calcium chloride per litre, pH 7.2.

2.2.2. Phosphate buffered saline

Oxoid PBS tablets 5
distilled water to 500ml.

This gave a solution equivalent to 0.8g sodium chloride, 0.2g potassium chloride, 0.15g disodium hydrogen orthophosphate dihydrate, 0.2g potassium dihydrogen orthophosphate per litre, pH 7.4.

2.2.3 Acetic ethanol fixative

glacial acetic acid 15cm^3

ethanol to 500ml.

2.2.4 Formol acetone fixative

potassium dihydrogen orthophosphate 0.1g

disodium hydrogen orthophosphate 0.02g

distilled water 30ml.

acetone 45ml.

formaldehyde solution 25ml.

2.2.5 Hypotonic 0.075M KCL solution

potassium chloride 5.59g

distilled water to 100ml.

2.2.6 Wolbach-Giemsa stain

Giemsa 1.0ml.

methanol 1.25ml.

0.5% sodium carbonate (aq.). 3 drops

distilled water 40ml.

2.2.7 Non-specific esterase stain

pararosanilin 1.0g

2M HCL 25ml.

sodium nitrite 4g

distilled water 100ml

Hexazotised pararosanilin was prepared according to the method of Barka & Anderson (1962).

Alpha Naphthyl acetate soln. :

alpha naphthyl actate 10mg

ethylene glycol monomethyl ether 0.5ml

2.3 INSTRUMENTS

2.3.1 Gas-Liquid Chromatography

Pye Unicam 204 - using a nitrogen detector and a Euroscribe recorder

For the detection of NMF, NEF, DBF and TMU :

Column - Carbowax 20M, 2 metre glass

Settings - oxygen 15 psi

hydrogen 12 psi

nitrogen 40 ml./minute

Column temp.- 185° C

Injector temp - 200° C

Detector temp - 200° C

For the detection of HMBA :

Column - 3% SP 2250 DB on 100/120 mesh Supelcoport, 2M glass.

Settings - oxygen 15 psi.
hydrogen 12 psi
nitrogen 40ml/minute.

Column temp. 220°C

Injector temp. 250°C

Detector temp 250°C

GLC was temperature programmed at 220°C for 5 minutes, rising by 4°C /minute to 236°C for 5 minutes.

2.3.2.Microscopes

Microscope - Nikon, Micro Instruments, Oxford.
x 10 eyepiece x 40 objective

Fluorescence microscope used a Nikon mercury lamp with a B20 filter system.

2.3.3. Computers

IBM PC and IBM AT using Lotus "Symphony" programme and the Statistical Graphics Corporation "Statgraphics" programme.

The programme used to calculate the "area under the curve" was kindly provided by Dr. J. Slack, Aston University, Birmingham. The LD_{10} was calculated using the Logic Analysis programme, which was a gift from Dr. B. Furr, ICI Pharmaceuticals, Macclesfield, Cheshire.

SECTION 3 METHODS

SECTION 3 METHODS

In Vitro Experiments

3.1 Cell Growth and Differentiation Assays

3.1.1. HL-60 Leukaemia Cell Growth

HL-60 promyelocytic leukaemic cells were provided by Mr. Tim Ward, Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester. They were grown by serial subculture every 3/4 days in 50 or 250ml plastic tissue culture flasks with RPMI-1640 medium containing L-glutamine and 25mM hepes, supplemented with 10% foetal calf serum (virus and mycoplasma screened). All tissue culture work was carried out in a Flow Laboratories Gelaire B5B3 laminar air flow cabinet. Each flask was gassed using a mixture of 10% CO2 in air and then incubated at 37°C. The cells were maintained in logarithmic phase of growth between $5x10^4$ and $5x10^5$ cells/ml. Cell number was determined with a Coulter Laboratories ZM or ZBI electronic cell counter.

3.1.2.L1210 Leukaemia Cell Growth

L1210 cells were provided by Flow Labs. They were grown by serial subculture every 3/4 days in 250ml plastic tissue culture flasks with RPMI-1640 medium supplemented with 10% horse serum. Each flask was gassed using a mixture of 5% CO2 in air and then incubated at 37°C. The cells were maintained in logarithmic phase of growth between $1x10^4$ and $5x10^5$ cells/ml.

3.1.3 Storage of Cells in Liquid Nitrogen

Cells growing in logarithmic phase were centrifuged in sterile universals for 5 minutes at 1500 rpm.in a Heraeus Labofuge 6000 centrifuge. Cell pellet was resuspended at $1x10^7$ cells/ml in cold 10% FCS/RPMI-1640 medium plus 10% dimethylsulphoxide. 1ml aliquots were dispensed into cryotubes and frozen slowly (at 2.3°C/min.) on ring C of a Linde BF-5 biological freezer in liquid nitrogen. The cells could be stored indefinitely at this temperature. When required, one cryotube was thawed rapidly using warm water and the contents added to 9ml of 10% FCS/RPMI-1640 medium. After 5-7 days incubation the cells were usually growing logarithmically.

Induction of Differentiation

The cells were diluted to $1x10^5$ cells/ml with 10% foetal

calf serum/RPMI-1640 medium and the appropriate volume of drug added. Where possible the equivalent volume of solvent alone was added to control flasks. The flasks were gassed and incubated at 37°C. Assays of differentiation were carried out daily or on days 4 and 7. The concentration of drug required for optimal differentiation was calculated from the equation:

Log Y = -0.0228m + 3.573 (Langdon and Hickman,1987).

where m is the molecular weight of the drug, and Y is the optimal differentiating concentration (mM).

The volume of polar solvent (such as NMF) added to the cells was calculated as follows:

$$\frac{\text{vol.of}}{\text{solvent}} = \frac{\text{required conc.}}{1000} \times \frac{\text{vol. in flask (ml)}}{1000} \times M_w \times \frac{1}{d}$$

where MW is the molecular weight of the solvent, and d is the density. Other solutions were made up as follows:

a) TPA and RPA

5mg of TPA (mol wt.617) or 5mg of RPA (mol.wt.700)

was dissolved in 5ml acetone. 100µl aliquots were pipetted into glass scintillation vials and mixture evaporated to dryness using nitrogen. The vials, each containing 100µl TPA or RPA were sealed, covered in foil and stored at -20°C in the dark. When required, the drug was redissolved in 10ml acetone and then either added directly to the cells (120µl in 20ml. to give a final concentration of 10^{-7}M) or further diluted to obtain concentrations of 10^{-8}M to 10^{-12}M TPA/RPA.

b) Retinoic Acid

Retinoic acid was stored in the dark at 4°C . When required 12mg was dissolved in 10ml methanol , this was further diluted to obtain a final stock solution containing 0.6µg retinoic acid/µl . This solution was used within 2 hours of preparation.

c)Hexamethylene Bisacetamide

500mg Hexamethylene bisacetamide was dissolved in 10ml sterilised distilled water or sterile saline. This was further diluted 1 in 1000 to give a final stock solution of 0.05µg/ul.

3.2. Assays of HL-60 Cell Terminal Differentiation.

3.2.1. Trypan blue exclusion assay for cell viability

1ml of cells at $1x10^5$ cells/ml were centrifuged in 1.5ml microcentrifuge tubes for 30 seconds at 11,600g using a Beckman microfuge B. The majority of the supernatent was removed and discarded. 5µl of 0.1% trypan blue dye in phosphate buffered saline (pH 7.4) was added to the cell pellet and this was mixed. One drop of cell suspension was added to a clean glass microscope slide and examined microscopically using either a Zeiss or Nikon Optiphot microscope with a 10x eyepiece and a 40x objective. Viable cells were able to exclude the dye and appeared colourless whereas non-viable cells had taken up the dye and appeared blue. A minimum of 300 cells were counted per sample, and the percentage of viable cells counted.

3.2.2. Nitrobluetetrazolium Reduction Assay

The nitrobluetetrazolium (NBT) solution was made up as follows:

10mg of NBT was dissolved in 10ml phosphate buffered saline, pH 7.4, to produce a saturated solution. 0.1mg of TPA was dissolved in 100µl acetone, and 50µl of this solution was added to the NBT solution.

$1x10^5$cells/ml were microfuged as before and the supernatent removed and discarded. 0.5ml of NBT solution was added to the cell pellet, mixed and then incubated in a shaking waterbath at 37^oC for 30-45 minutes. The cells were then centrifuged at 11.600g, the majority of the supernatent removed and one drop of cell suspension was placed on a clean glass slide and examined microscopically. Cells that had differentiated to either neutrophil or monocyte/macrophage cells were stimulated by the TPA to produce an oxidative burst yielding superoxide which then reduced the yellow NBT to blue/black formazan granules, clearly visible under the light microscope. Undifferentiated cells remained colourless. A minimum of 300 cells were scored and the percentage of differentiated cells calculated.

3.2.3.Determination of Phagocytic Capacity

a)Preparation of complement-coated dyed yeast particles

Yeast cells were prepared according to the method of Shaala (1979) as follows:

5-6g of bakers yeast were washed twice with PBS, centrifuging for 5 minutes at 5000rpm., and resuspended in 50ml PBS and autoclaved. The yeast was washed with PBS

until the supernatent remained clear, 7/8 times, and incubated at 37°C for 2 hours in 25ml PBS containing 0.17ml ß-mercaptoethanol. Yeast were then washed once with 0.9% saline, resuspended in 50ml of 0.02M iodoacetamide in PBS, pH 7.4, and incubated at room temperature for 2 hours, checking that the pH remained constant. Yeast were washed twice with PBS, autoclaved and then washed again until the supernatent remained clear. The number of yeast particles was determined using a haemocytometer and they were resuspended in 0.9% saline at $2x10^9$ cells/ml. 120ml saline containing 260mg procion rubine was added to this suspension and the yeast were incubated at room temperature for 3 hours. The excess dye was removed by washing with PBS and the yeast were resuspended at $2x10^9$ cells/ml with 20mg sodium azide in 20ml PBS and stored at 4°C.

The yeast surface has, at this stage been reduced and alkylated and the particles dyed red. To ensure efficient uptake into the cells, the yeast particles were coated with complement.

2ml of red dyed yeast suspension was added to 8ml of complement fixation buffer and 2ml of rabbit serum, the mixture was then incubated at 37°C for 30 minutes. The suspension was washed twice with RPMI-1640 medium and resuspended in 20ml medium (approx. $5x10^8$ cells/ml). The

yeast suspension was then aliquoted into 0.5ml portions in bijoux bottles and stored at -20°C.

b)Assay for Phagocytosis

$1x10^5$ HL-60 cells were centrifuged at 11,600g as previously described and the supernatent removed and discarded. The cell pellet was mixed with a 1:4 mixture of RPMI-1640 medium and hepatitis-free AB^+ human plasma and 15µl of dyed yeast suspension. Cells were incubated at 37°C for one hour, then centrifuged and the supernatent removed. 4µl of 0.1% toluidene blue in saline was added to counterstain any yeast cells external to the HL-60 cells, one drop was placed on a glass microscope slide and the mixture was examined microscopically. Cells which had differentiated to either granulocytes or monocyte/macrophages, and therefore possessed the C3b complement receptor, were able to phagocytose the complement-coated yeast cells and these cells contained one or more red yeast particles. A minimum of 300 cells were scored and the percentage of cells capable of phagocytosis was calculated.

3.2.4. Staining for Non-Specific Esterase

$1x10^5$ HL-60 cells were centrifuged as before and the

majority of the supernatent removed and discarded. Smears were made of the remaining cell suspension on glass microscope slides and air-dried for 2-5 minutes. The smears were fixed for one minute with formol acetone fixative, rinsed using distilled water and air-dried for 30 minutes. Slides were stained, according to the method of Yam et al, (1971), at 37°C for 1.5 hours in a buffered pararosaniline solution which was made up as follows:

Hexazotised pararosaniline was prepared according to the method of Barka and Anderson (1962). 1.5ml of sodium nitrate was mixed with 1.5ml of pararosaniline in 2M hydrochloric acid one minute prior to use. 45ml phosphate buffered saline was first mixed with 2.5ml of alpha-naphthylacetate in ethylene glycol monomethyl ether and the hexazotised pararosaniline solution added. The staining solution was thoroughly mixed, the pH adjusted to 5.8-6.2 using 1M sodium hydroxide solution and the stain was filtered prior to use.

After staining, slides were washed in running water, counterstained for one minute with 0.1% methyl green in distilled water, washed for a further 1-2 minutes and allowed to air dry before being examined microscopically. Cells that had differentiated to monocytes or macrophages appeared red, undifferentiated cells appeared green. A minimum of 300 cells were scored and the percentage of monocytic cells was determined.

3.2.5.Morphological Staining using Wolbach Giemsa Stain

Smears were made of a cell suspension as previously described (3.2.4.), air dried and fixed for 2-5 minutes in acetic-ethanol fixative. Smears were stained in a solution containing 1ml Giemsa, 1.25ml methanol, 3 drops 0.5% sodiun carbonate solution and 40ml distilled water. The staining solution was replaced three times during the first hour with fresh stain and the slides were incubated in the final staining solution overnight at room temperature. The slides were differentiated using 70% ethanol, taken to water, mounted using xam mountant, and examined microscopically. A minimum of 300 cells were scored, they were considered differentiated if the nucleus was "kidney-shaped" or the nuclear : cytoplasmic ratio <1. Undifferentiated cells had a large round nucleus and the nuclear:cytoplasm > 1.

3.2.6.Staining with Monoclonal Antibodies

a)Preparation of slides

A modification of the method by Huang et al (1983) was employed. Glass slides were prepared by evenly placing 6 drops of a 50:50 glycerol:water mixture onto

alcohol-cleaned slides. The slides were sprayed for 30 seconds using Klingerflon spray, allowed to air dry for 2-3 minutes and the glycerol washed off using running water. Slides were air dried at room temperature for 15 minutes before being immersed in a 0.05% solution of poly-L-lysine for 10 seconds - this has been shown to improve cell adhesion for immunocytochemistry. Slides were air dried for 20 minutes and stored at room temperature in cardboard boxes.

b)Use of Monoclonal Antibodies

$1x10^5$ HL-60 cells were microfuged for 30 seconds, the supernatent removed and discarded and the cell pellet resuspended in 2-3 drops of 100% foetal calf serum. One drop of cell suspension was placed on each indentation on the prepared slide and removed to create a very thin film of cells on the slide surface. The slide was air dried and, if necessary, could be stored in the dark at $-20^{\circ}C$ until required. 50µl of a 10% FCS/PBS solution was added to a 5µl aliquot of monoclonal antibody and mixed using a whirlimixer. 5µl of this solution was placed on each indentation on the slide and incubated at room temperature for one hour in a sealed plastic box lined with damp paper. Slides were washed using PBS for 2-3 minutes and the area surrounding each indentation carefully dried using a tissue to prevent the fluorescent conjugate spreading over the slide. 5µl of fluorescent conjugate

was mixed with 100µl of 10% FCS/PBS mixture and 5µl of the resulting solution placed onto each indentation and the slides were incubated at room temperature in the humid box for 30 minutes. On removal, the slides were washed for 2 minutes using PBS and counterstained using 0.05% ethidium bromide in PBS for 30-60 seconds. Slides were finally washed for one hour in PBS before being mounted in xam and examined microscopically using a Nikon fluorescent microscope.

The cells which possessed the OKM1 antigen, which is the C3b complement receptor, appeared green and undifferentiated cells appeared red/orange. A minimum of 300 cells were scored and the percentage of differentiated cells calculated.

3.3.1. Colony Formation in Semi-Solid Agar

A modification of the method used by Tew and Wang (1982) was employed. HL-60 cells were centrifuged for five minutes at 6000g, and resuspended in the cloning mixture of 10% FCS/RPMI-1640 medium at a cell density of 500 cells/ml. 2ml aliquots were pipetted into 4.5cm petri dishes and stored at 37°C. 22ml RPMI-1640 medium was prewarmed in a waterbath to 44°C. A presterilsed 5ml aliquot of 5%^{W}/v Difco Noble agar was boiled once to melt and allowed to cool to 60°C. 3ml of agar was dispensed into 22ml medium at 44°C and 3ml aliquots were pipetted

into the petri dishes containing cells. The petri dishes were shaken gently to mix the contents, the final amount of agar was 0.36%. The agar was allowed to set for 5-10 minutes and the petri dishes were incubated in plastic boxes at 37°C in a gassing incubator and examined for clones after 9-12 days.

3.4.1. Measurement of Commitment to Differentiate

a) HL-60 cells were diluted to $1x10^5$ cells/ml using 10% FCS/RPMI-1640 medium. The various inducers of differentiation were added at their optimum differentiating concentrations (defined in section 4.1) and, where possible, an equivalent volume of solvent was added to the control cells. All the flasks were gassed and incubated at 37°C. At various time intervals, between 1/2-24 hours, one incubate was collected and the cells washed free of drug 3 or 6 times by centrifuging for 5 minutes at 1200 rpm. The control cells were also washed at various timepoints and the cell pellet was resuspended in 20ml of fresh medium without the inducer. At 72 hours from the start of the experiment, all the flasks were shaken gently to dislodge any attached cells. Assays of viability and differentiation (NBT, phagocytosis and NSE) were carried out to determine the length of incubation with the various inducers that was required to irreversibly commit a significant proportion of the cells

to differentiate even after removal of the inducer.

Then experiments were carried out to determine the commitment time required when 170mM NMF, 1.0mM BDF, 80mM NEF, 1.0mM TMBF, 2mM HMBA, 1nM TPA and 1nM RPA were used.

b)To determine if TPA/RPA was being washed out from the cells, bioassays of the supernatents were also carried out by resuspending 10^5 untreated HL-60 cells in 10ml of the supernatent obtained from each wash. These cells were also assessed for differentiation 72 hours from the start of the experiment.

c)To determine whether the time required for commitment using TPA/RPA could be decreased, experiments were carried out in which the cells were pretreated with 10nM retinoic acid for 18 hours prior to the addition of 1nM TPA/RPA. The cells were washed 3 times to remove the retinoic acid before the inducer was added and the commitment procedure was repeated.

3.4.2. Alternate Regimes of dosing and washing HL-60 cells in vitro

To attempt to reproduce the *in vivo* pharmacokinetics of NMF, NEF, TMU and HMBA in *in vitro* conditions throughout a 4 day regime of dosing, various washing and dosing regimes

were performed. The HL-60 cells were exposed to the drugs for variable time periods throughout the four days with the cells being washed free of the drugs for the remaining time intervals.

a)Experiments using the optimum differentiating concentrations of the drugs

HL-60 cells were diluted to a density of $1x10^5$ c./ml using fresh medium. Drugs were added to the cells to give concentrations of 170mM NMF, 80mM NEF, 8.5mM TMU or 2mM HMBA. After 12 hours the cells were washed three times by centrifugation and were resuspended, at the same density, in fresh medium for a further 12 hours. This proceedure was repeated for four consecutive days with the drugs having been present for 12 out of every 24 hours. 12 hours after the final dose of drugs had been removed the cells were assessed for viability and differentiation.

b)Experiments using the maximum concentration of drug obtained in vivo.

HL-60 cells were diluted to a density of $1x10^5$c./ml using fresh medium. Drugs were added to the cells to give concentrations of 7mM NMF, 5mM NEF, 12mM TMU and 6mM HMBA,

which were the maximum concentrations of the drugs that were measured *in vivo*. The washing and refeeding proceedure described in 3.4.2.a. was repeated, the cells being assessed for viability and differentiation on the fifth day.

c)*Experiments using a drug exposure equivalent to the in vivo AUC value*

The drug levels obtained in diffusion chambers, which had been implanted into mice for 5 days, were determined and the AUC values were calculated for NMF, NEF, TMU and HMBA over a 24 hour time period. The exposure time of each drug required to give an equivalent AUC value was subsequently calculated for 6 hour exposure times. These concentrations of NMF, NEF, TMU and HMBA were added to HL-60 cells *in vitro* , at a density of $1x10^5$c./ml, for 6 hours/day for 4 days, with the cells being washed free of drug as described in 3.4.2.a. The viability and differentiation were then assessed 18 hours after the last dose of drug had been removed from the cells.

d)*Experiments using the maximum concentration of TMU obtained in vivo and an equivalent AUC value*.

The maximum concentrations of TMU and HMBA that were obtained in diffusion chambers *in vivo* were determined. These concentrations of TMU and HMBA were added to HL-60

cells, at a density of $1x10^5$c./ml, for an equivalent exposure time as the AUC value. That is, 12mM for 3.5 hours/day for TMU and 5mM HMBA for 1hr 40 mins./day. The inducers were added for 4 consecutive days, with the cells being washed free of the inducers as described in 3.4.2.a. Differentiation and viability were assessed on day 5.

3.5.1. Differentiation Induction by the Simultaneous Addition of TPA and Retinoic Acid.

HL-60 cells were diluted to a density of $1x10^5$ c./ml with fresh medium. The cells were then either : a) pretreated with 10nM retinoic acid for 18 hours, washed 3 times by centrifuging for 5 minutes at 1200 rpm, and 1nM TPA added; or, b) treated with 1nM TPA; or, c) treated simultaneously with both 10nM retinoic acid and 1nM TPA. Control cells were treated with an equivalent volume of solvent. All the flasks were gassed and incubated at 37°C for 3 days before being counted and assessed for differentiation.

3.6. Measurement of the Growth of HL-60 cells and L1210 cells in Diffusion Chambers in vitro

Diffusion chambers were constructed using 0.45, 0.22, 0.1µm filter membranes. 150µl of HL-60 cells, with or without 10% FCS, at a density of $5x14^4$c/ml or L1210 cells

at a density of $5x10^4$ c./ml. were injected into each chamber and the chambers were sealed using molten wax. The chambers were incubated at 37°C in sterile universals containing 3ml of gassed medium with or without 10% FCS. The cell number, viability and percentage of differentiation was assessed daily by removing the chamber, breaking open the filter membrane using a Pasteur pipette and removing the contents.

IN VIVO STUDIES

3.7.1. Construction of Diffusion Chambers

Diffusion chambers were constructed in a tissue culture screen by cementing Millipore filters to a 13mm Lucite ring, 2mm thick, using Millipore MF cement no.1. Completed chambers were sterilised by irradiation (100,000 rads) prior to use and stored in 250ml. plastic tissue culture flasks.

3.7.2. Implantation of Diffusion Chambers

Sterile diffusion chambers were filled with 150µl of cell suspension and the filling orifice was sealed using 1-2 drops of molten wax. The chambers were stored in

sterile universals containing 5ml of gassed RPMI-1640 medium with/without 10% FCS immediately prior to use.

CBC/CA mice were anaesthetised using 0.1ml of a 10% solution of Hypnorm and 5mg/kg Diazepam. The area surrounding the incision site was clipped using dissecting sissors and shaved to remove as much superfluous fur as was possible. A 1-1.5cm incision was made in each mouse, and the chamber implanted in the peritoneal cavity. The wound was sutured using Ethicon suture silk and securely sealed using 3 Michel clamps. The mice were allowed to recover under a lamp for warmth and then randomly divided into appropriate treatment groups.

3.7.3.Removal of Diffusion Chambers

Mice were killed by cervical dislocation, the peritoneal cavity opened and the chambers removed using spoon forceps. The chambers were wiped free of adherent mouse tissue and incubated in 0.025% pronase solution at 37°C for 30 minutes to lyse the plasma clot within the chamber. After this time, the chambers were blotted dry and the contents removed by rupture of the filter membrane using a sterile Pasteur pipette. The cell suspension volume was adjusted to 150µl using RPMI-1640 before assays of differentiation were carried out.

3.8. Characterisation of the Diffusion Chamber System as a Model of Drug-Induced Terminal Leukaemic Cell Differentiation.

3.8.1. Alteration of the Chamber Implantation Site.

Chambers containing HL-60 cells were either implanted adjacent to a centrally positioned incision site or they were implanted distal to a lateral incision site. The growth and differentiation of HL-60 cells was assessed over a 7/10 day time period.

3.8.2. Alteration of Initial Cell Number Injected into the Chamber.

The chambers were constructed using 0.45μm filters, and a lateral incision site was used. The initial density of HL-60 cells injected into the chamber was $5x10^5$, $1x10^6$, and $5x10^6$ cells/ml.; the initial density of *in vitro* L1210 cells was $5x10^5$, and $2x10^6$ cells/ml., and the initial density of *in vivo* L1210 cells was $5x10^5$, $2x10^6$ and $1x10^5$ cells/ml.. The growth characteristics of L1210 and HL-60 cells and the spontaneous differentiation of HL-60 cells was assessed over a 10 day time period.

3.8.3. Varying the Filter Pore Size.

The chambers were constructed using 0.1, 0.22 and 0.45μm pore size membrane filters. HL-60 cells were used at an initial density of $1x10^6$ cells/ml and the initial density of *in vivo* L1210 cells was $5x10^5$ cells/ml . Chambers were implanted using a lateral incision site. The growth characteristics of HL-60 and L1210 cells and the spontaneous differentiation of HL-60 cells was assessed over a 10 day time period.

3.8.4. Growth of HL-60 cells in Diffusion Chambers in the presence and absence of 10% Foetal Calf Serum.

Serum-free cells were obtained by centrifuging 10ml HL-60 cells at a density of $1x10^6$ c./ml for 5 minutes at 1200 rpm. The supernatent was removed and discarded. The cell pellet was resuspended in 10ml. RPMI-1640 medium and centrifuged again. This procedure was repeated 3 times and the cell pellet finally resuspended in 10ml RPMI-1640 medium. 150μl of cell suspension, either with or without serum was injected into each diffusion chamber as previously described (3.7.2.). The growth characteristics and the percentage of spontaneous differentiation of HL-60 cells was assessed over a 21 day time period.

3.10. LD 10 Determination of N-ethylformamide, tetramethylurea, dibutylformamide, tetramethylbutylformamide and hexamethylene bisacetamide.

The lethal dose 10% was defined as that dose of drug which killed 10% of the mice when administered as a four day regime of dosing and the observation time was for 7 days after the final dose was administered. The maximum tolerated dose of a drug was defined as the maximum dose of drug that could be administered for 4 consecutive days without causing the death of any of the animals; these were calculated using the computer programme (see page 102).

Ten mice were injected at each dose of drug for 4 consecutive days. The doses used were 700, 600, 500, 400 mg/kg NEF; 2200, 2000, 1800, 1700, 1650 mg/kg TMU; 300, 200, 150, 100 mg/kg DBF; 20, 15, 12.5 mg/kg TMBF and 3500, 3000, 2750, 2500 mg/kg HMBA. The mice were observed for 7 days after the final injection and the number of deaths that occurred at each dose were recorded.

3.11. Pharmacokinetic Studies

3.11.1. Gas Liquid Chromatographic Analysis of Drugs in Blood and Diffusion Chambers .

Standard solutions of 100, 200, 300, 400 and 500µg/ml of NMF, NEF, DBF and TMU were prepared when required.

100µl of each standard solution, chamber contents or heparinised blood was added to 900µl of acetone containing the internal standard. For analysis of NMF, NEF and DBF the internal standard was 15µl/ml TMU; and for analysis of TMU the internal standard was 100µg/ml NMF. The solutions were thoroughly mixed and microcentrifuged in a Beckman microfuge B for 30 seconds at 11,600g. 1.5µl of the supernatent was injected onto the GLC column, the oven and column temperature settings as described in 2.3.1.

For analysis of hexamethylene bisacetamide, the method described by Litterst et al (1985) was used. The standard solutions were 100-5000µl/ml and the internal standard was 1mg/ml N-(3-(dimethylamino)propylbenzamide. 200µl of the internal standard was added to 100µl aliquots of plasma, chamber contents or HMBA standard solutions. Samples were microfuged using a Beckman microfuge B for 1 minute and the supernatent was assayed directly on the GLC, the settings as described in 2.3.1.

The amount of drug was determined by calculating the ratio of the peak height of the drug to the peak height of the internal standard. The calibration curve of this ratio against each drug concentration was plotted using the "Statgraphics" program on an IBM PC computer and the unknown drug concentrations were determined from this calibration curve using the "Statgraphics" computer program.

3.11.2. Pharmacokinetics of a single dose of NMF, NEF, TMU and HMBA and DBF over a 24 hour time period.

CBA/CA mice implanted with chambers that had been resident for either 1 or 5 days were used. The chambers were constructed using 0.45μm filter membranes and contained either 150μl of serum-free HL-60 cells at a density of $1x10^6$ cells/ml or 150μl of RPMI-1640 medium alone.

The mice were given a single ip. injection of 80% of the LD 10 dose of the drugs: 400mg/kg NMF, 350mg/kg NEF, 2020mg/kg HMBA, 1600mg/kg TMU. All drugs were diluted using saline, and the DBF was diluted using saline and tween 80. At 1, 2, 4, 8, 12, and 24 hour time intervals the mice were killed under anaesthetic by cardiac puncture and the blood which was removed was stored at -20°C in 1.5ml heparinised microcentrifuge tubes until required. The diffusion chambers were removed from the mice and wiped free of any adherent mouse cells/tissue. The filter membranes were broken open using a Pasteur pipette and the contents were transferred to a microcentrifuge tube and stored at -20°C until GLC analysis could be carried out. The area under the curve was calculated for each drug using the trapezoidal rule on the computer program.

3.11.3.A 4 Day Regime of Dosing of NMF, NEF, HMBA, TMU and DBF to Determine whether the drugs accumulate within the chamber.

Diffusion chambers which were constructed from 0.45μm filter membranes and containing 150μl of serum-free HL-60 cells at a density of $1x10^6$ cells/ml were implanted into CBA/CA mice. On day 5, the mice were given a single ip. injection of 400mg/kg NMF, 350mg/kg NEF, 2020mg/kg HMBA, 1600mg/kg TMU or 150mg/kg DBF. After 24 hours, 2 mice from each treatment group were killed under anaesthetic by cardiac puncture and the chamber contents removed for analysis. The remaining mice were injected again ip. with either NMF, NEF, HMBA, TMU or DBF. 24 hours after the final injection, the remaining 2 mice from each treatment group were killed under anaesthetic and the chamber contents removed. The blood and chamber concentrations of all the drugs were assayed by GLC (as described in section 3.11.1)

3.11.4.Determination of the localisation of DBF in vivo

CBA/CA mice were injected with a single ip. dose of 200mg/kg. dibutylformamide. After 2 hours the mice were killed by cervical dislocation. The liver and adipose

tissue were removed and weighed. DBF was extracted from these tissues by homogenising the liver and adipose tissue with 5ml PBS on ice. 15ml of dichloromethane was added to each homogenate and the contents mixed thoroughly. 2-6g of anhydrous sodium sulphate was added to remove the water and the homogenates were filtered. The filtrate was evaporated to dryness using a rotary evaporator, the water bath temperature was 45°C, for 30-60 minutes. The contents were reconstituted in 100µl acetone and assayed by GLC using the settings as described in 2.3.1. Liver and adipose tissue were removed from untreated control mice and spiked with 100µl/ml DBF to ensure that the extraction procedure was efficient.

3.12. In Vivo Differentiation

Diffusion chambers, which were constructed from 0.45µm filter membranes and containing 150µl of serum-free HL-60 cells at a density of $1x10^6$ cells/ml, were implanted into CBA/CA mice. On day 5, 6, 7 and 8, the mice were weighed and then given a single ip. injection of 80% of the LD 10 dose dose of NMF, NEF, TMU, DBF or HMBA. 24 hours after the final injection, the mice were killed by cervical dislocation, the chamber removed and the contents assessed for differentiation by assays of NBT, NSE and monoclonal antibodies.

SECTION 4 RESULTS AND DISCUSSION

SECTION 4 RESULTS and DISCUSSION

4.1. Characterisation of Differentiation Induced by Various Differentiating Agents

a) N-Methylformamide(NMF)

Incubation of HL-60 cells with 50-250mM NMF was found to induce the terminal differentiation of the cells to granulocytes as was previously determined by Collins et al (1978) and Langdon & Hickman (1987) (Fig 4.1.).

The untreated cell population divided approximately 4.4 times over the 5 day period suggesting a population doubling time of 28 hours. This was an average time for HL-60 cells as cell doubling times of as short as 16-20 hours have been reported (Graham et al, 1985), and the original cell line, which was isolated from the patient and cultured in an enriched medium containing 20% FCS and conditioned medium, had an initial population doubling time of 55-60 hours (Collins et al, 1977). Increasing the concentration of NMF reduced the extent of cell proliferation with total inhibition at 250mM NMF. Cell viability decreased significantly at concentrations

FIG.4.1. Experiments to determine the relationship between the induction of differentiation and various concentrations of N-methylformamide.

Fig 4.1a - Cell numbers measured on days 3 and 5
Fig 4.1b - Differentiation measured on day 3.
Fig 4.1c - Differentiation measured on day 5.

All figures are the mean values of 4 experiments and the standard error <5%

Fig 4.1a

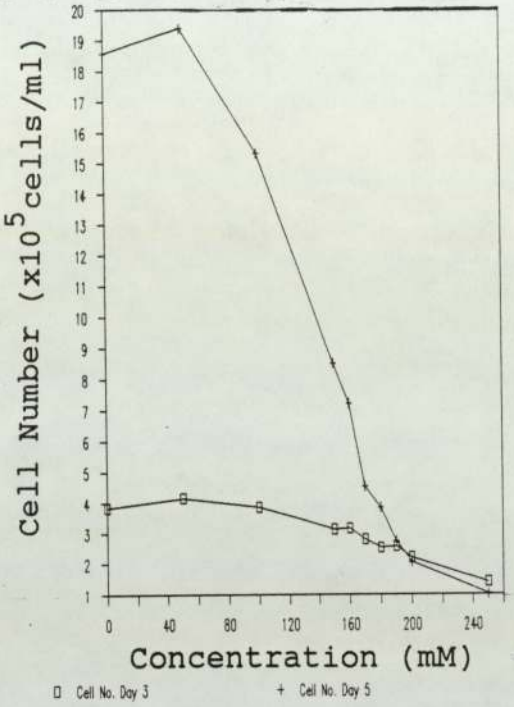


Fig 4.1b

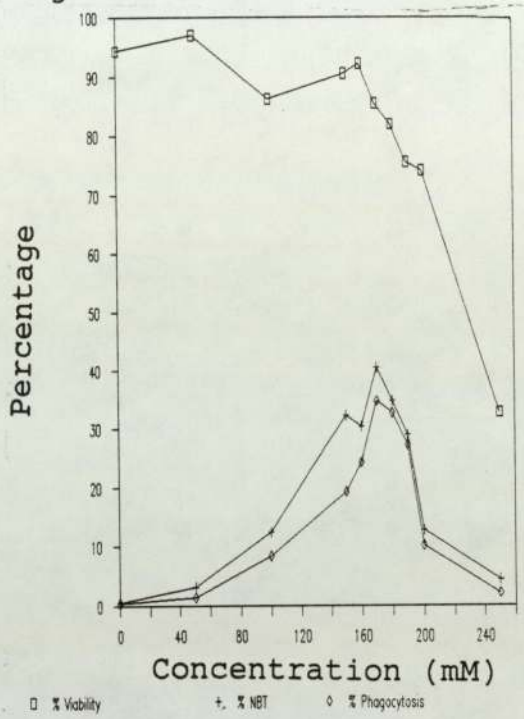
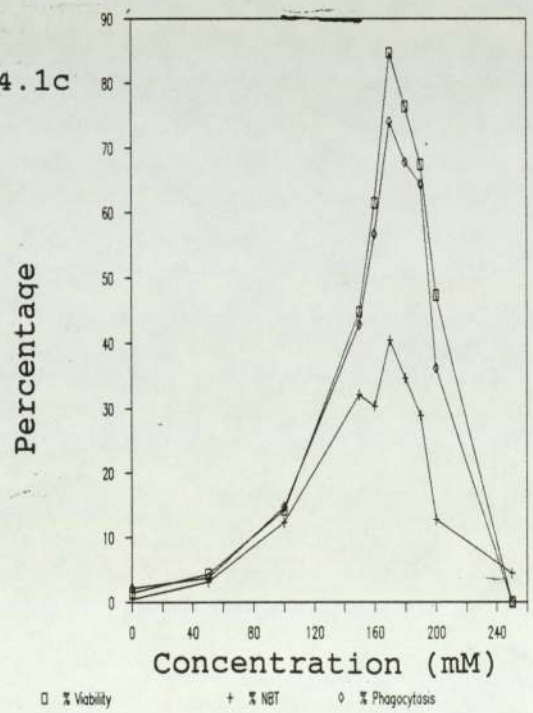


Fig 4.1c



greater than 180mM NMF, with only 30% of the remaining cells viable at a concentration of 250mM.

The extent of cell differentiation induced by NMF was also concentration-dependent with less than 10% of cells induced to differentiate with 50mM NMF, which increased to maximum of >80% of cell differentiation after treatment with 170mM NMF. At concentrations above 170mM, the percentage of cells induced to differentiate decreased as cell viability decreased. The optimum concentration of NMF for the induction of differentiation - defined as that concentration which allowed a maximum percentage of cells to express both biochemical and functional characteristics of differentiation whilst generally retaining a viability of >85% - was 170mM NMF, which was in concordance with the value determined by Langdon & Hickman (1987).

In further experiments, this optimum concentration of NMF was used to determine the temporal relationship between addition of the inducer and the expression of biochemical and functional markers of differentiation (Fig 4.2.). After 1 day of treatment, cell proliferation and other markers of differentiation were unaffected, but by day 2, proliferation was inhibited, as compared with control cells, and thereafter increased only slowly so that by day 7 only 2.5 population doublings had occurred, compared with 4 doublings of the control cells. There was no significant induction of NBT-reducing activity or

FIG.4.2.Assay of the effects of 170mM NMF on the differentiation of HL-60 cells at various time periods.

Fig 4.2a - Cell numbers- control and NMF-treated cells.
Fig 4.2b - Percentage of differentiation of the NMF-treated cells
Percentage of differentiation of the control cells was <8% and the cell viability was >90%. Data not shown.

All figures are the mean values of 4 experiments and the standard error <5%

Fig 4.2a

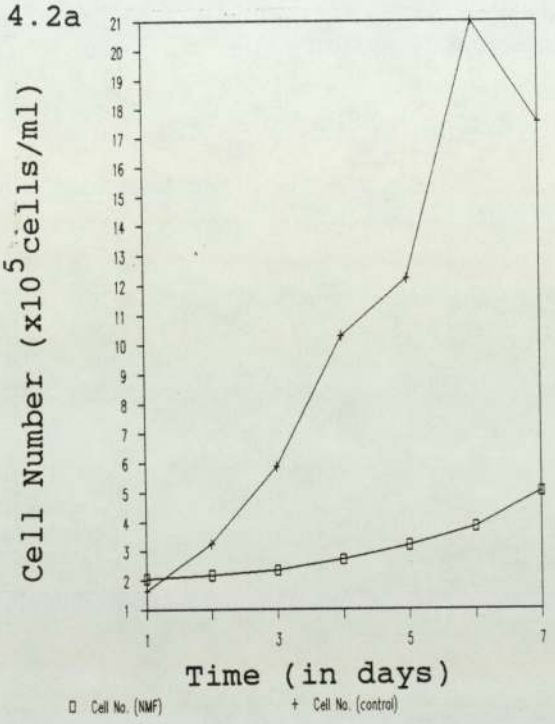
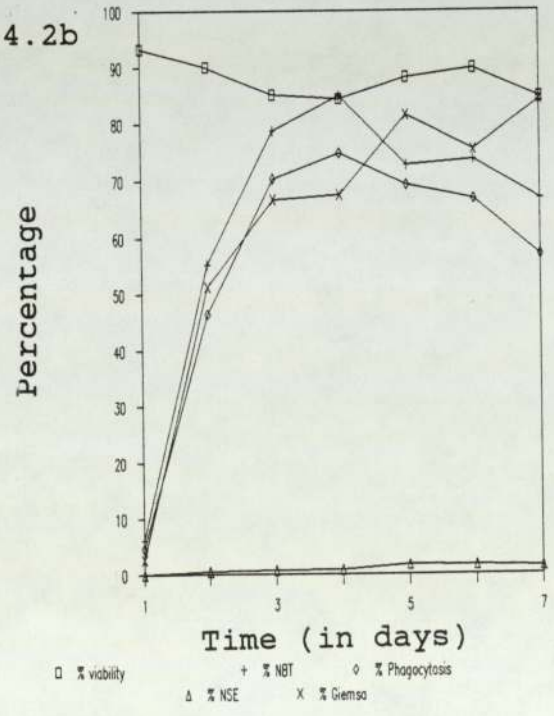


Fig 4.2b



induction of the ability of the cells to phagocytose until day 2 and the percentage of cells expressing these markers reached a maximum by day 4 and thereafter declined by 20% by day 7. The cell viablity was greater than 85% for the duration of the experiment.(Fig 2·1 – 2·3).

The requirement for at least one cell division for the development of the differentiated phenotype was not surprising: normal promyelocytes undergo division in order to give rise to the more differentiated myelocytes and metamyelocytes. During normal granulopoiesis the cells do not lose their proliferative ability until the metamyelocyte stage is reached, and the subsequent maturation to banded and segmented neutrophils occurs in the absence of proliferation (Bessis, 1973).

Yen et al (1986) have stated that growth arrest was a prerequisite for phenotypic differentiation; and Fibach et al (1982) and Von Melchner & Hoffken (1985) have indicated, from experiments in which HL-60 cells were cloned after treatment with the inducer, that the loss of self-renewal potential of the cells preceded their commitment to differentiate. None of these groups explained why this inhibition should be necessary to trigger the cells to differentiate.

A two step hypothesis for the induction of differentiation has thus been proposed (Fibach et al, 1982., Yen et al,

Fig. 2.1. Photograph to show terminally differentiated HL-60 cells stained for non-specific esterase.

HL-60 cells were treated with 1nM TPA for 4 days before being stained for non-specific esterase. The cells that have terminally differentiated to monocyte/macrophage cells appear red whereas the undifferentiated cells appear green.

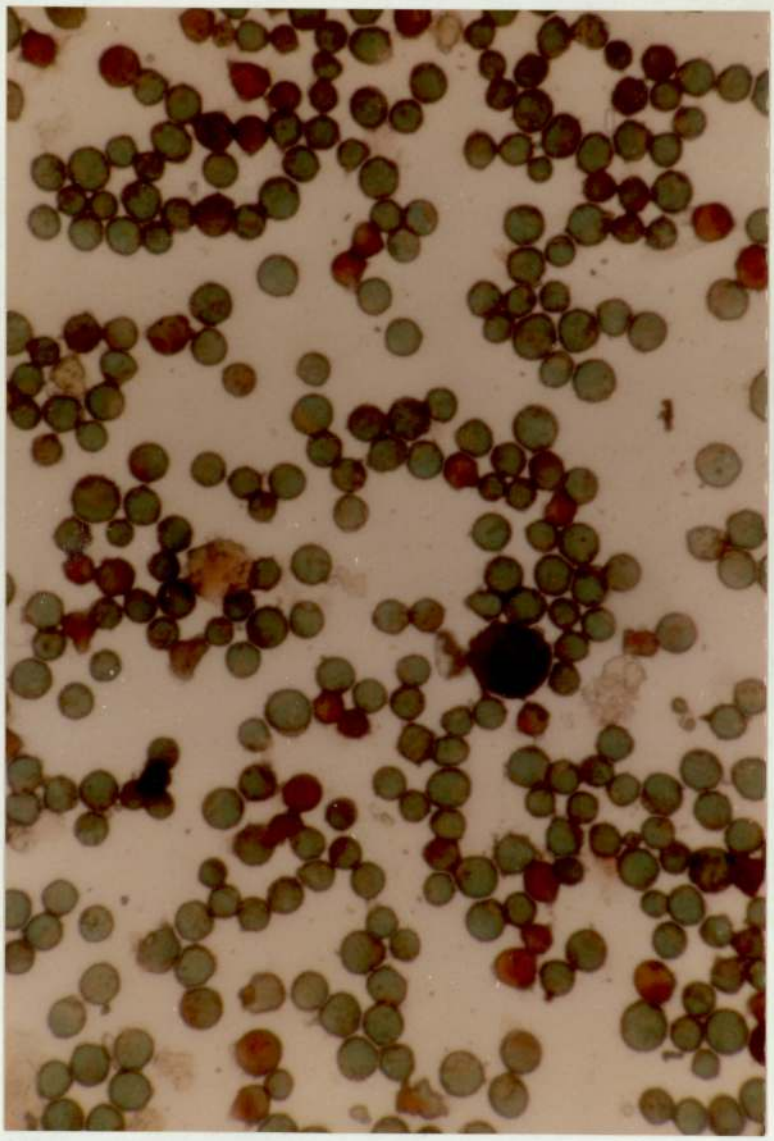


Fig. 2.2. Photograph to show the results of the assay of phagocytosis using terminally differentiated HL-60 cells.

HL-60 cells were treated with 170mM NMF for 4 days before being assessed for phagocytic ability. Cells that contained 2 or more of the dyed yeast particles were scored as positive. Any yeast particles external to the cells are counterstained blue.

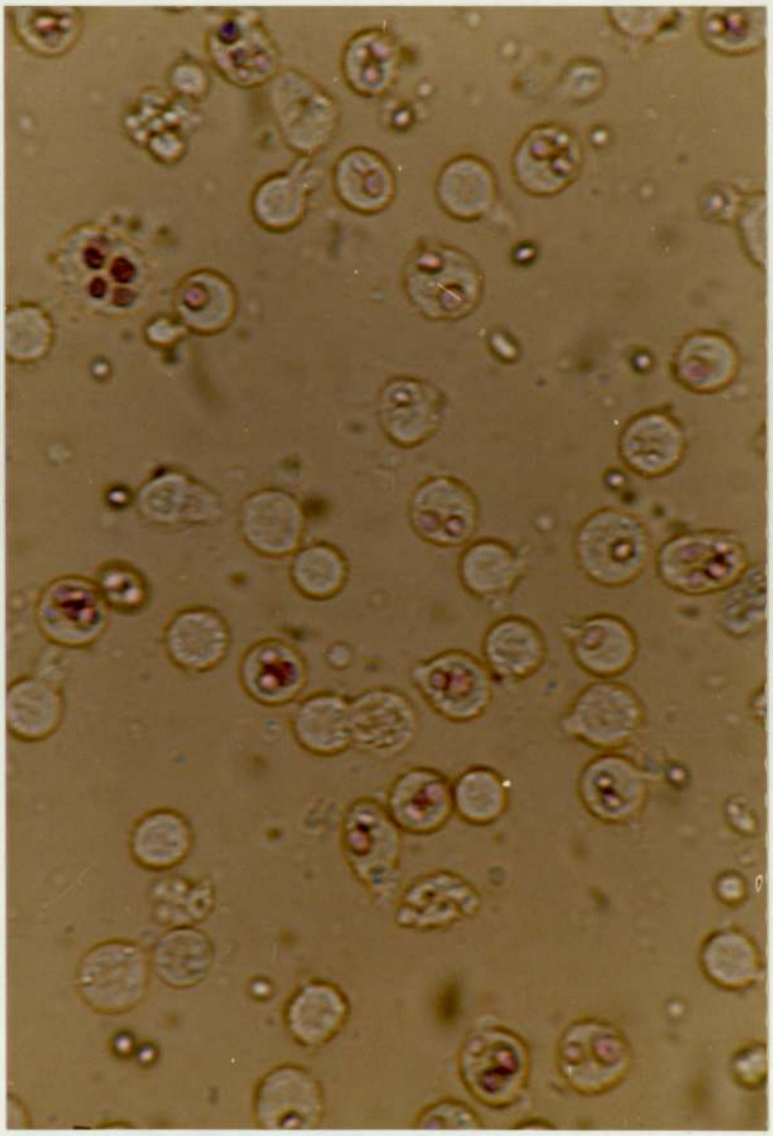
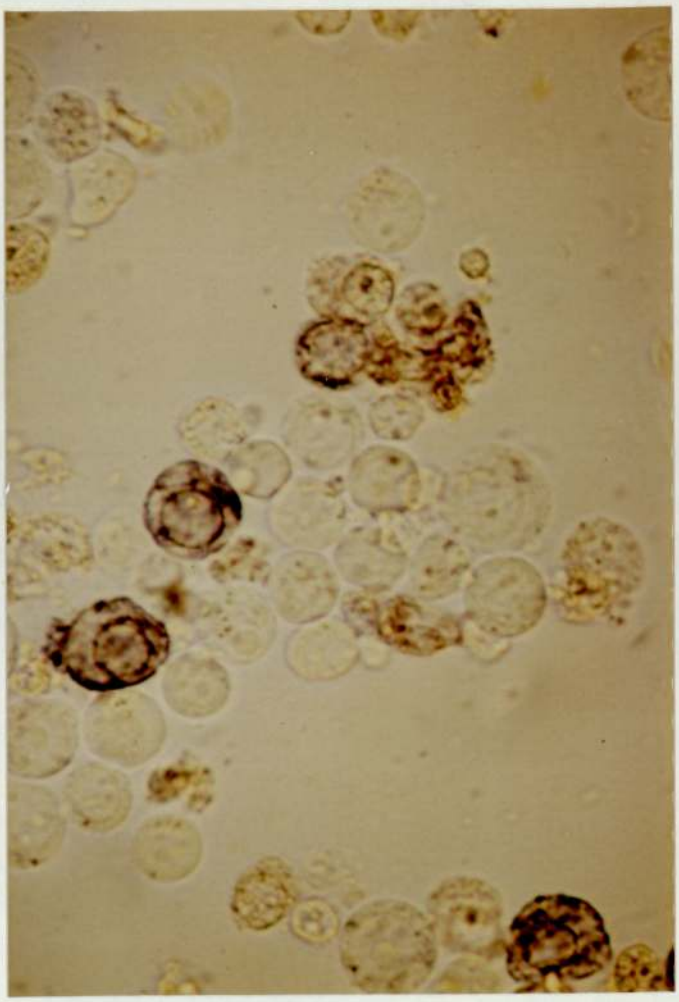


Fig. 2.3. Photograph to show the results of the assay of NBT using terminally differentiated HL-60 cells.

HL-60 cells were treated with 170mM NMF for 4 days before being assessed for the ability to reduce NBT. The cells that had terminally differentiated were able to reduce the yellow NBT to purple formazan granules - which were visible within the cells. The undifferentiated cells appeared colourless.



1985). Yen et al (1986) showed that di-cis isomers of retinoic acid were able to complete the early events leading to growth arrest but were unable to complete the later events leading to phenotypic differentiation which occurred with all the other isomers of retinoic acid studied. The two events were therefore separable, although the late differentiating inducing events did not occur unless preceded by the events leading to growth arrest. Both Fibach et al (1982) and Von Melchner & Hoffken (1985) showed that the early loss of self-renewal potential was followed by events leading to irreversible commitment to differentiation, which reinforced the hypothesis that the induction of terminal differentiation in HL-60 cells is a two step process.

Yen (1987) subsequently showed that the first step associated with growth arrest occurred whether the cells were treated with a granulocytic or monocytic inducer and that only in the second stage of commitment was the lineage determined.

Incubation of HL-60 cells with 170mM NMF induced greater than 75% of cells to differentiate, but, interestingly, there was always a fraction of cells that did not differentiate. This fraction of cells was not considered to be a stably resistant sub-population and some supporting evidence for this was provided by Boyd & Metcalf (1984). They isolated the few clones of HL-60 cells that did not

differentiate in response to butyrate, and recloned these cells in butyrate. They found the same heterogeneity of response as in the original sample, suggesting that those cells which did not differentiate in the original sample were not resistant to the induction of differentiation.

To confirm that NMF had induced the cells to differentiate to granulocytes, monocytic non-specific esterase (NSE) activity was determined in these cells. This remained virtually undetectable over the 7 day period. The granulocytic appearance was confirmed by staining the cells with Wolbach Giemsa. Many cells were seen to have either a reniform nucleus, characteristic of metamyelocytes, or a banded nucleus which was characteristic of mature granulocytes, whereas the undifferentiated promyelocytes had a large round nucleus. By day 7, a significant proportion of the cells had a lobulated nucleus characteristic of neutrophils.

b) N-Ethylformamide (NEF)

Incubation of HL-60 cells with NEF was also shown to induce terminal granulocytic differentiation as determined by Langdon & Hickman (1987) (Fig 4.3.). The control cell population divided approximately 4 times over the 5 day period studied, whereas increasing concentrations of NEF

FIG.4.3.Experiments to determine the relationship between the induction of differentiation and various concentrations of N-ethylformamide.

Fig 4.3a - Cell numbers measured on days 3 and 5.
Fig 4.3b - Differentiation measured on day 3.
Fig 4.3c - Differentiation measured on day 5.

All figures are the mean values of 4 experiments and the standard error <5%

Fig 4.3a

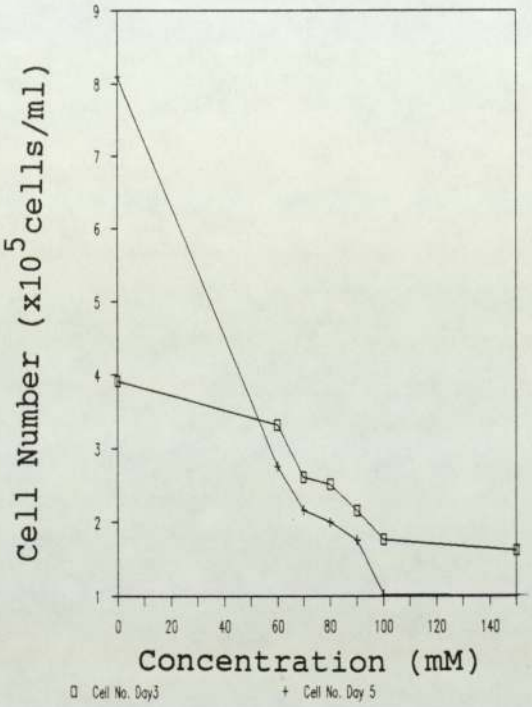


Fig 4.3b

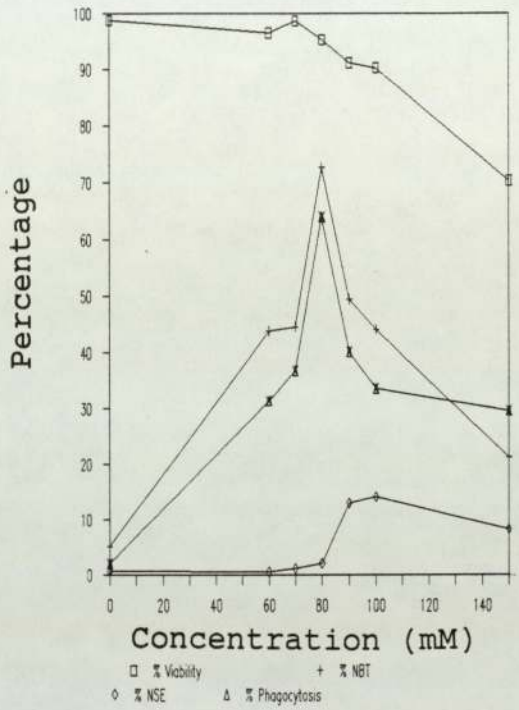
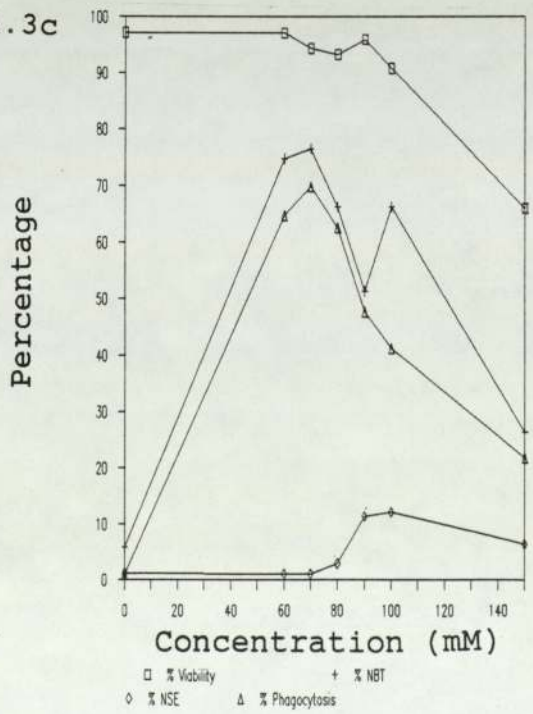


Fig 4.3c



inhibited cell proliferation, with total inhibition at 150mM NEF. Cell viability decreased significantly at concentrations greater than 110mM. The optimum concentration of NEF required for differentiation was determined to be 80mM, this concentration induced significant differentiation whilst retaining a cell viability of >85%. This optimum concentration was in agreement with the expected optimum differentiating concentration calculated from the equation by Langdon & Hickman (1987).

Examination of the temporal relationship between addition of the inducer and expression of markers of differentiation was investigated using a concentration of 80mM NEF. The expression of such markers was very similar to that observed with NMF (Fig 4.4.). No significant NBT reducing activity was measurable until day 2; this increased to a maximum of 84.16% by day 4. The percentage of cells capable of phagocytosing complement-coated yeast also increased to a maximum of 77.29% by day 4. The percentage of NBT-positive cells was always greater than the percentage of cells with phagocytosis activity (measured on the same day) suggesting that the expression of the C3b complement receptor is a late rather than an early marker of differentiation.

Surprisingly, there was a small but significant increase in the monocytic NSE activity, which is usually specific

FIG.4.4.Assay of the effects of 80mM NEF on the differentiation of HL-60 cells at various time periods.

Fig 4.4a - Cell numbers, control and NEF-treated cells.
Fig 4.4b - Percentage of differentiation of the NEF-treated cells.
Percentage of differentiation of the control cells was <8% and the cell viability was >90%. Data not shown.

All figures are the mean values of 4 experiments and the standard error <5%

Fig 4.4a

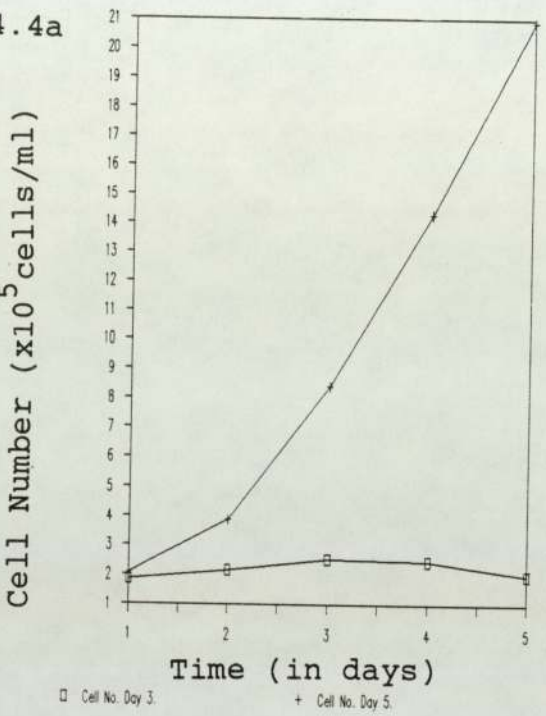
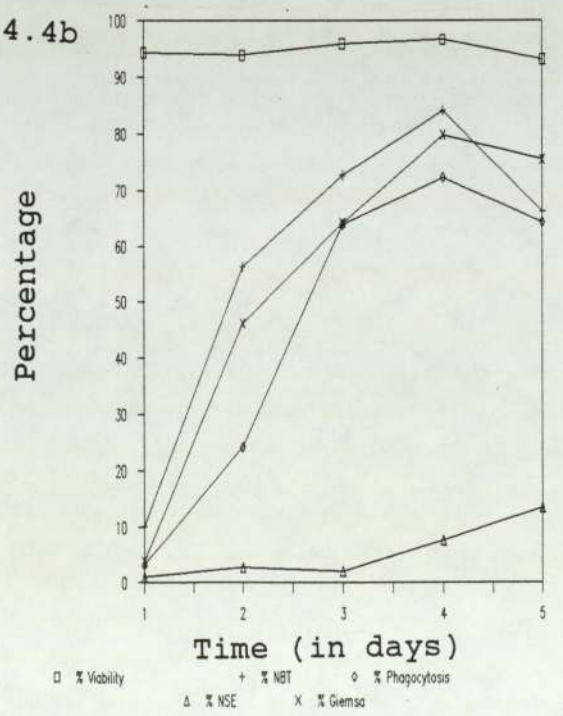


Fig 4.4b



for monoctyic/macrophage cells; this increased gradually and reached a maximum by day 5. The differentiated cells did not adhere to the tissue culture flasks or attain the flattened morphology characteristic of macrophages (HL-60 cells induced to differentiate to macrophages by the phorbol ester TPA have this characteristic morphology) (Fibach et al, 1984). The cells that were expressing markers of NSE were probably more similar to the non-adherent monocytes which resulted from HL-60 cell differentiation in response to ara-C (Ross, 1985). However, examination of the nuclear morphology, assayed by Wolbach Giemsa stain, suggested that the majority of the cells were in fact granulocytes.

Previous studies by Yen et al (1987) had determined that the early events in commitment were common to both granulcyte and macrophage pathways although it was unclear how NEF could cause the cells to differentiate along both pathways simultaneously. A possible explanation for this phenomenon was suggested by Rose et al (1986) (as outlined in section 1.3.1). They proposed that progenitor cells, committed to differentiation towards megakaryocytes, erythrocytes, neutrophils, monocytes, T & B cells were arranged in a linear sequence and thus it was possible for the promyelocytic cells to differentiate to either granulocytes or monocyte/macrophage cells. This hypothesis was in contrast to the branched pathway of differentiation (Fig 1.2) which suggested that

promyelocytic cells should only be capable of differentiation towards granulocytes and not to monocyte/macrophage cells.

c) Tetramethylurea (TMU).

TMU had been demonstrated by Langdon & Hickman (1987) to be a potent inducer of the terminal differentiation of HL-60 cells. Incubation of HL-60 cells with TMU produced a concentration-response curve as shown in Fig 4.5.

The percentage of cells which expressed markers of terminal differentiation increased in a concentration-dependent manner with an optimum concentration for differentiation of 8mM. There was again a significant increase in NSE activity, which reached a maximum of 17% on day 3 with 10mM TMU. Interestingly, there were increased amounts of NSE activity at the more toxic concentrations of TMU than at the lower concentrations. The percentage of cells capable of the reduction of NBT increased to a maximum of 79.83% by day 5, suggesting that although the majority of cells had differentiated to granulocytes, as judged by their nuclear morphology, NBT and phagocytic activity, a small percentage had differentiated to macrophage-like cells and expressed NSE activity.

FIG.4.5 Experiments to determine the relationship between the induction of differentiation and various concentrations of Tetramethylurea.

Fig 4.5a - Cell numbers measured on days 3 and 5.
Fig 4.5b - Differentiation measured on day 3.
Fig 4.5c - Differentiation measured on day 5.

All figures are the mean values of 4 experiments and the standard error <5%

Fig 4.5a

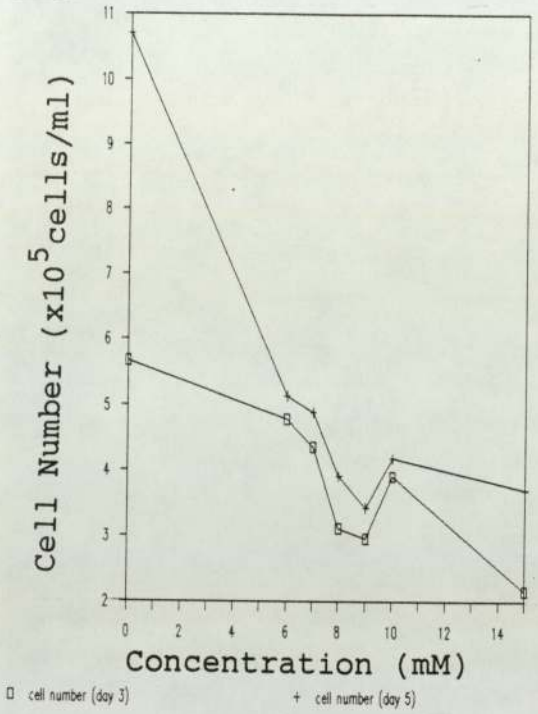


Fig 4.5b

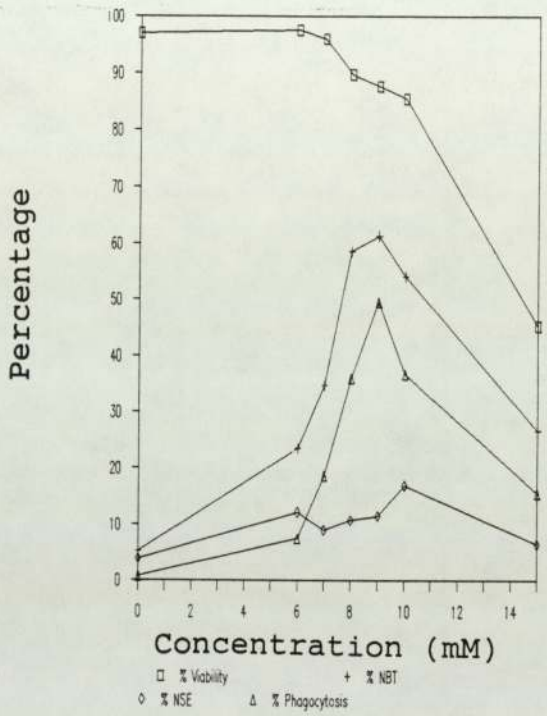
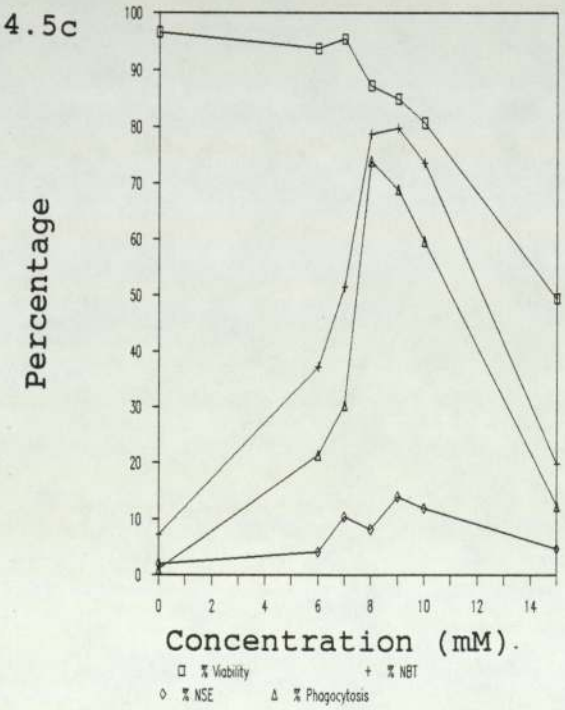


Fig 4.5c



The time course for the induction of differentiation was then assessed using 8mM TMU (Fig 4.6). This was again very similar to that observed for NMF. The cells divided 1.5 times during the 5 day period whilst the untreated cell population doubled 4.5 times. The cell viability remained in excess of 85% for the duration of the experiment.

There was no significant induction of cells expressing markers of terminal differentiation after 1 day of treatment with TMU, but by day 2 there was significant increases in the number of cells capable of the reduction of NBT and the phagocytosis of yeast particles. These reached a maximum on day 4, with a lag in NSE activity which reached a maximum on day 5 (see section 4.1a).

d)Dibutylformamide and Tetramethylbutylformamide (DBF and TMBF)

Characterisation of the in vitro differentiation induced by these formamides was studied as they were considered possible candidate molecules for the induction of terminal differentiation in vivo. Work reported by Langdon & Hickman (1987) had suggested that the molecular weight of the inducer was an important feature of its differentiating ability; the higher the molecular weight the less of the inducer was required to induce

FIG.4.6 Assay of the effect of 8.5mM TMU on the differentiation of HL-60 cells at various time periods.

Fig 4.6a - Cell numbers- control and TMU-treated cells.
Fig 4.6b - Percentage of differentiation of the TMU-treated cells.
Percentage of differentiation of the control cells was <8% and the cell viability was >90%. Data not shown.

All figures are the mean values of 4 experiments and the standard error <5%

Fig 4.6a

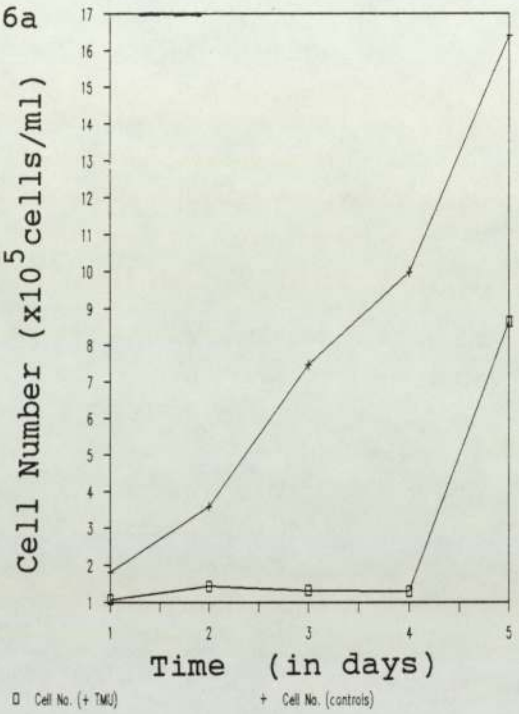
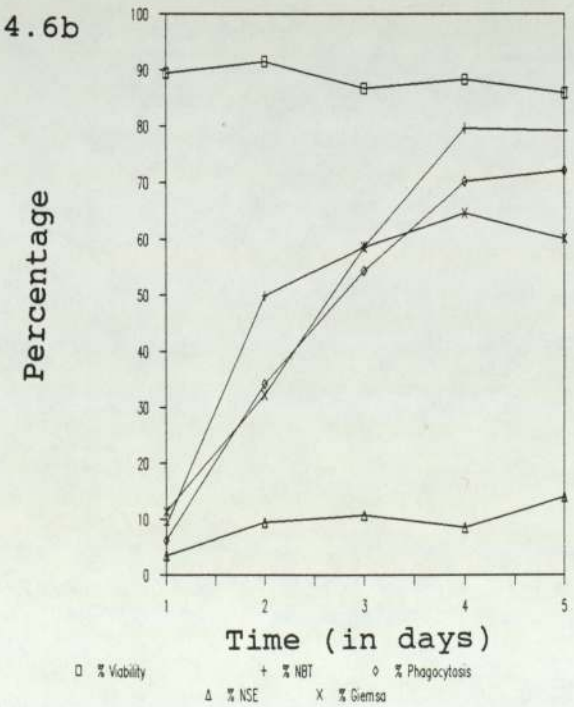


Fig 4.6b



differentiation. It was therefore decided to examine some formamides of higher molecular weight than NMF to determine whether they could induce the terminal differentiation of HL-60 cells in vitro.

Both these formamides showed very similar concentration-response curves (Figs 4.7 and 4.8.) which was not surprising as they both have the same molecular weight. The percentage of differentiation induced was concentration-dependent and increased up to an optimum of 61% at 1mM before decreasing concomitant with decreasing cell viability. Even at the optimum differentiating concentration of 1mM, the percentage of NBT positive cells only reached 61% compared with 84% induced by 170mM NMF. Increasing the concentration of these inducers resulted in a decreased cell viability without an increase in differentiation. There were also a significant percentage of cells expressing NSE activity which was again significantly higher at the toxic dose levels than at the lower, less toxic, concentrations. This suggested that some of the cells had been induced to differentiate to monocyte/macrophage cells whilst the majority had been induced to differentiate to granulocytes.

There was much variation in the efficiency of the inducers of differentiation: NMF and NEF induced differentiation in more than 75% of cells, whereas DBF and TMBF could only induce 60% of the cells to differentiate. This variation

FIG.4.7.Experiments to determine the relationship between the induction of differentiation and various concentrations of N-dibutylformamide.

Fig 4.7a - Cell numbers measured on days 3 and 5.
Fig 4.7b - Differentiation measured on day 3.
Fig 4.7c - Differentiation measured on day 5.

All figures are the mean values of 4 experiments and the standard error <5%

Fig 4.7a

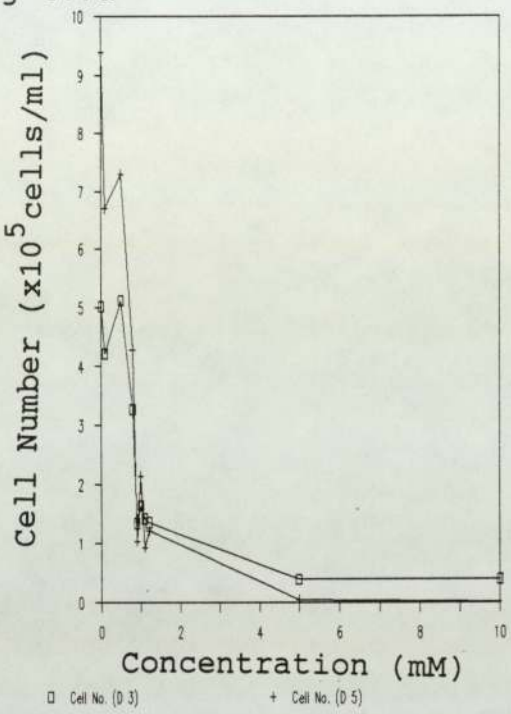


Fig 4.7b

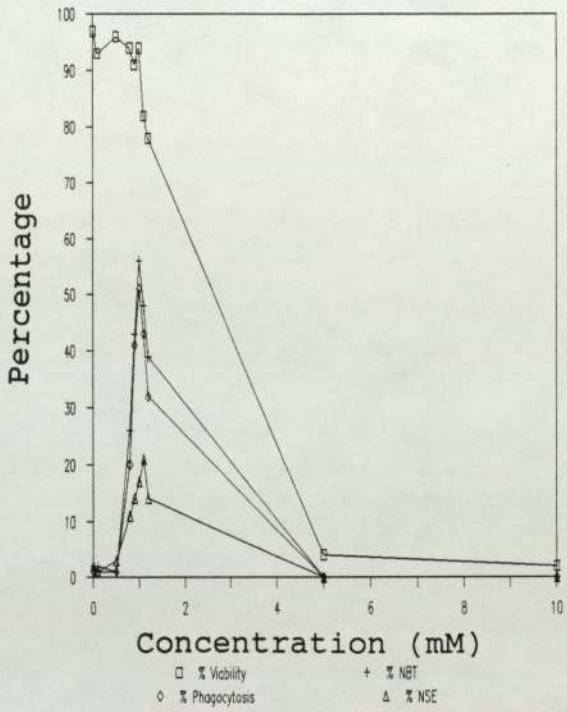


Fig 4.7c

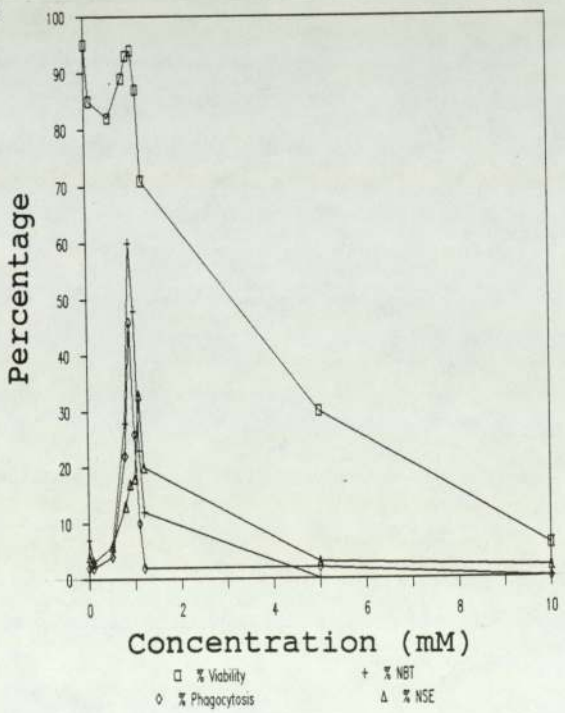


FIG.4.8.Experiments to determine the relationship between the induction of differentiation and various concentrations of Tetramethylbutylformamide.

Fig 4.8a - Cell numbers measured on days 3 and 5.
Fig 4.8b - Differentiation measured on day 3.
Fig 4.8c - Differentiation measured on day 5.

All figures are the mean values of 4 experiments and the standard error <5%

Fig 4.8a

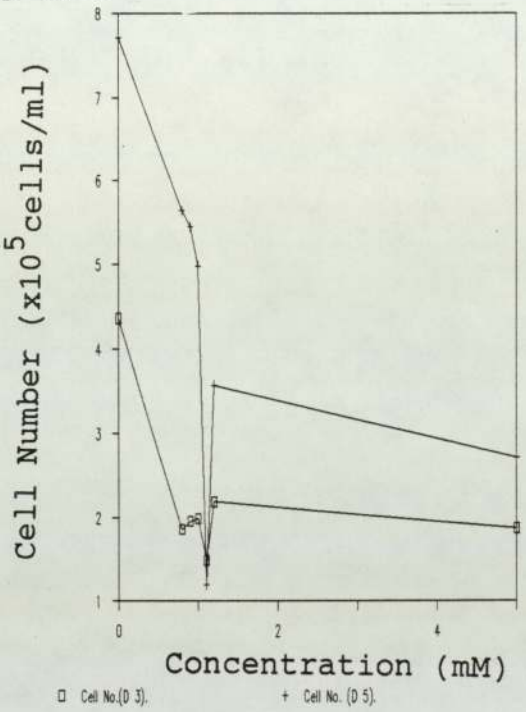


Fig 4.8b

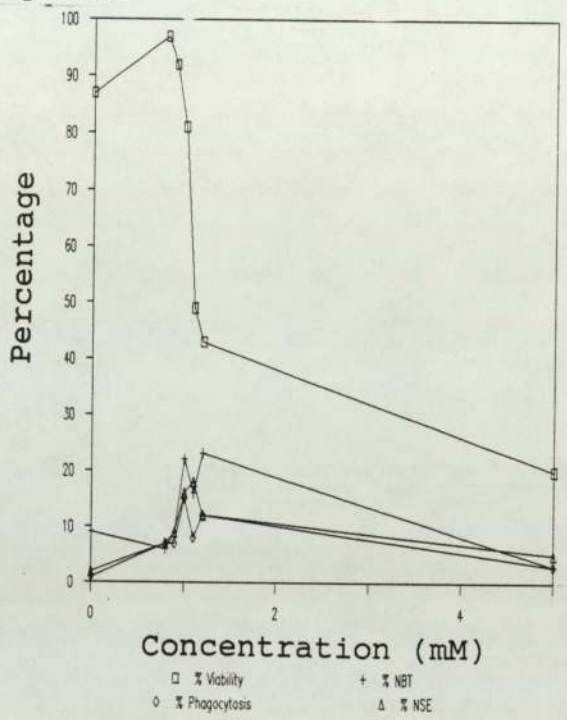
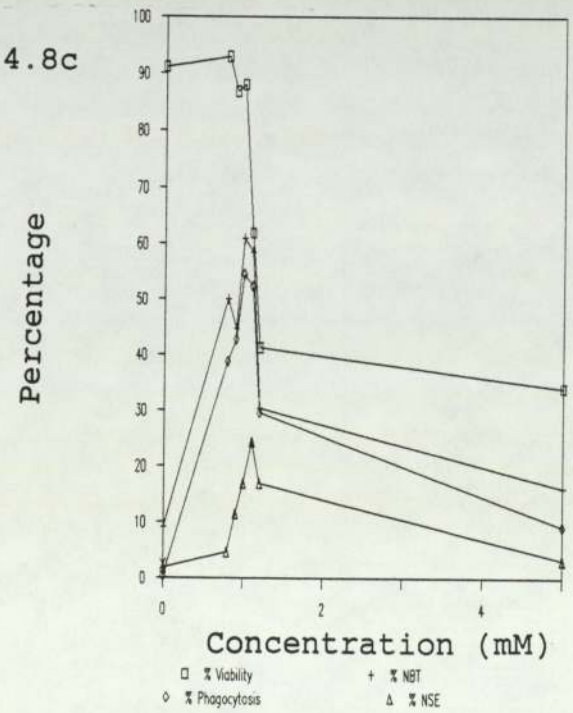


Fig 4.8c



in the efficiency of the inducers has been noted before: Langdon & Hickman (1987) reported that whilst acetone and dimethylforamide induced more than 70% of HL-60 cells to differentiate, even at the optimum concentraion formamide could induce no more than 40% and urea only induced 11%. A suggestion was made that agents such as formamide were rapidly metabolised in the cells so that the intracellular concentration was not maintained for long enough for commitment of the majority of the cells to occur.

These in vitro results were in contrast to the in vivo experiments to determine the LD_{10} of these agents (Table 4.1). DBF was considerably less toxic in vivo than TMBF. The mice treated with toxic doses of DBF usually died 1-3 days after a 4 day dosing regime, suggesting some type of chronic damage had been incurred by the inducer. On histological examination of the livers of the DBF-treated mice a marked change in structure was noted (data not shown). In contrast, injection of TMBF caused some mice to enter spasm and some not and, if it occurred, it resulted in death; if it did not occur the mice survived and no histological abberrations were found in sections of these livers (data not shown).

Daily assessment of differentiation showed the markers of differentiation being induced after 1 day of incubation with either inducer as with NMF (Figs 4.9 and 4.10). The optimum concentrations required for the induction of

Table 4.1. LD_{10} Value of NEF, DBF, TMBF and HMBA.

Inducer	LD_{10} (mg/kg)	80% LD_{10}
NEF	440	350
DBF	190	150
TMBF	15	12.5
HMBA	2525	2020

All mice were dosed with the drug for 4 consecutive days and the observation period was 7 days after the final injection.

FIG.4.9. Assay of the effect of 1mM DBF on the differentiation of HL-60 cells at various time periods.

Fig 4.9a - Cell numbers- control and DBF-treated cells.
Fig 4.9b - Percentage of differentiation of the DBF-treated cells.
Percentage of differentiation of the control cells was <8% and the cell viability was >90%. Data not shown.

All figures are the mean values of 4 experiments and the standard error <5%

Fig 4.9a

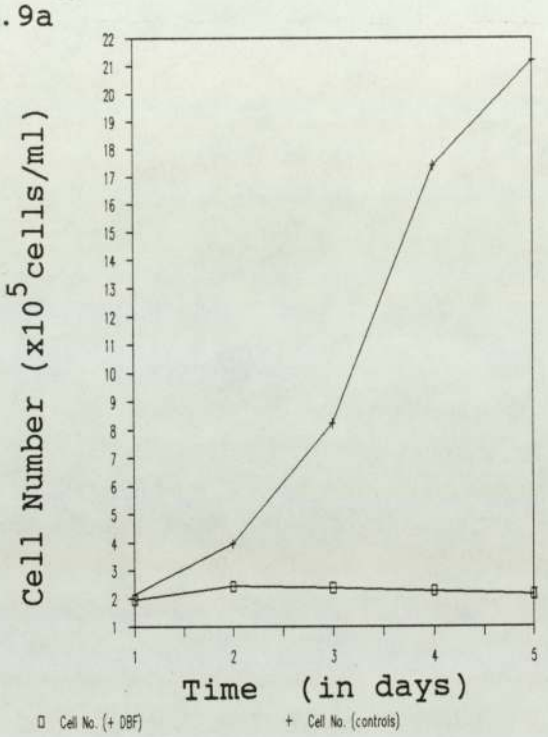


Fig 4.9b

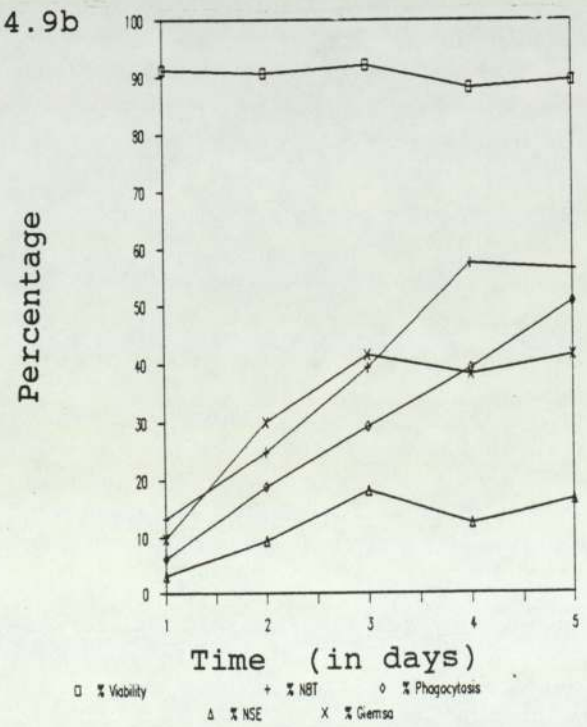


FIG.4.10.Assay of the effect of 1mM TMBF on the differentiation of HL-60 cells at various time periods.

Fig 4.10a - Cell numbers- control and TMBF-treated cells.
Fig 4.10b - Percentage of differentation of the TMBF-treated cells.
Percentage of differentiation of the control cells was <8% and the cell viability was >90%. Data not shown.

All figures are the mean values of 4 experiments and the standard error <5%

Fig 4.10a

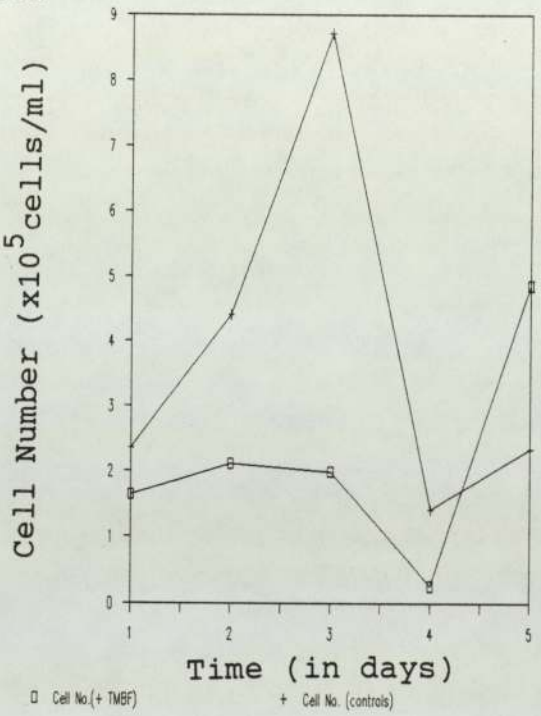
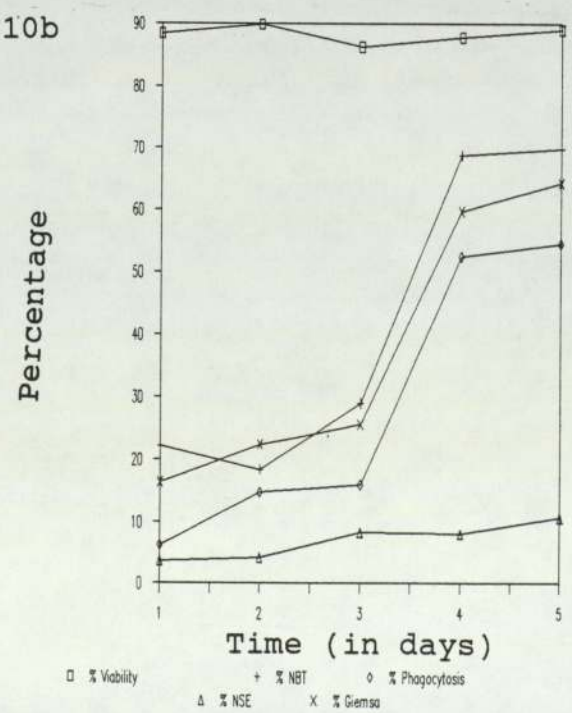


Fig 4.10b



differentiation *in vitro* were 1mM for both DBF and TMBF, and the percentage of differentiation was at a maximum by day 4.

e) Hexamethylene bisacetamide (HMBA)

The optimum concentration for the induction of differentiation of HL-60 cells was determined to be 2mM HMBA, which was in agreement with that reported by Collins et al (1978) (Fig 4.11). At this concentration the cells were still in excess of 90% viable after 5 days and 84% of cells were capable of reducing NBT. None of the HMBA-treated cells expressed markers of NSE activity at any of the concentrations used. At concentrations below 2mM, the percentage of differentiation increased in a dose-dependent manner; and at a concentration of 3mM and above, the percentage of differentiation decreased concomitantly with decreasing cell viability.

Examination of the temporal relationship between the addition of the inducer and the expression of markers of differentiation using 2mM HMBA showed similar characteristics to those observed with the previous inducers of granulocytic differentiation (Fig 4.12). The cells expressed increasing NBT and phagocytic activity after 2 days treatment which increased to a maximum by day

FIG.4.11.Experiments to determine the relationship between the induction of differentiation and various concentrations of Hexamethylene bisacetamide.

Fig 4.11a - Cell numbers measured on days 3 and 5.
Fig 4.11b - Differentiation measured on day 3.
Fig 4.11c - Differentiation measured on day 5.

All figures are the mean values of 4 experiments and the standard error <5%

Fig 4.11a

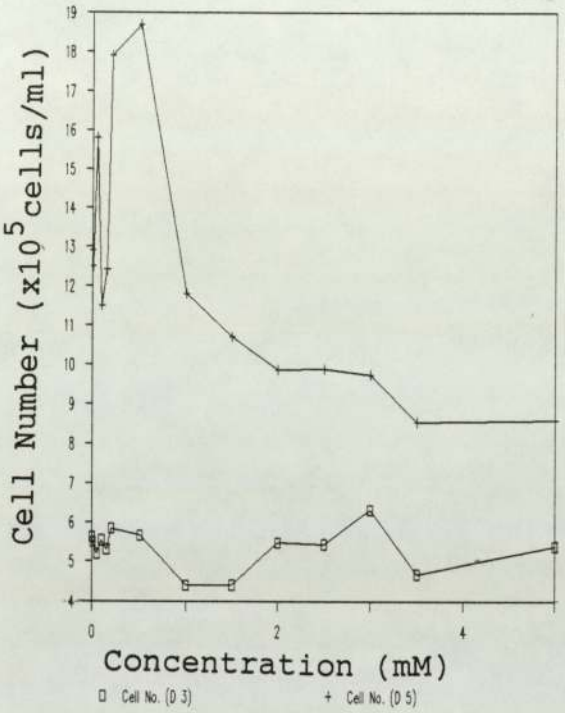


Fig 4.11b

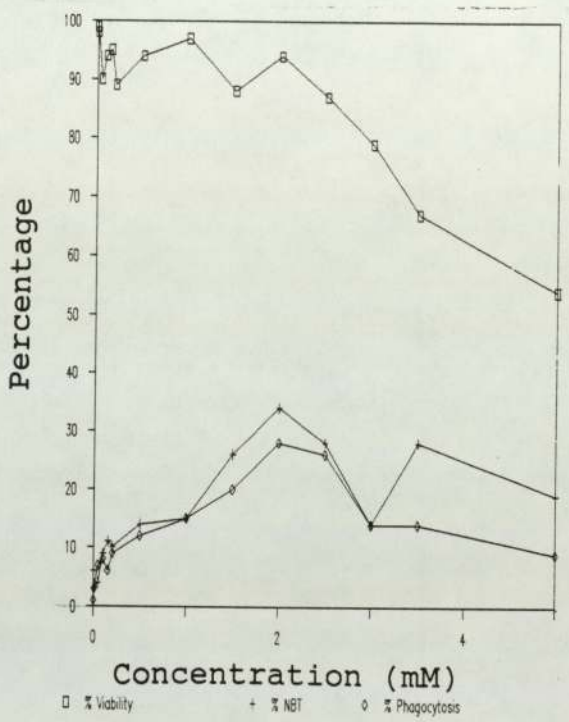


Fig 4.11c

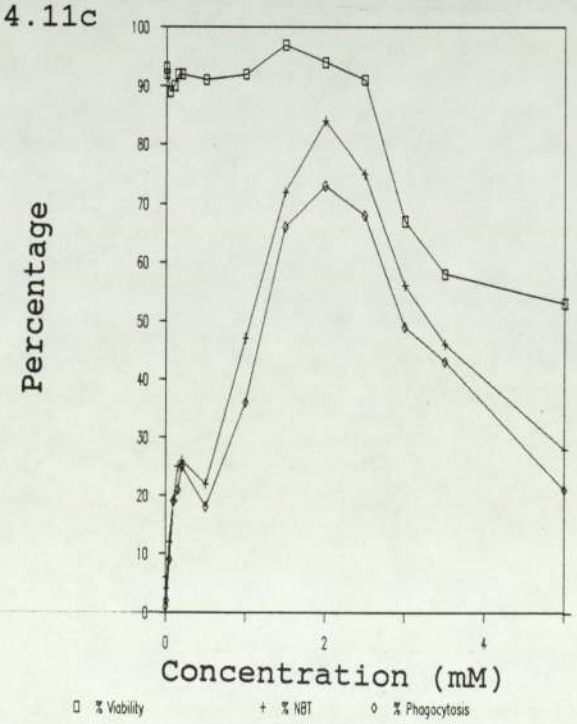


FIG.4.12. Assay of the efffect of 2mM HMBA on the differentiation of HL-60 cells at various time periods.

Fig 4.12a - Cell numbers, control and HMBA-treated cells.
Fig 4.12b - Percentage of differentiation of the HMBA-treated cells.
Percentage of differentiation of the control cells was <8% and the cell viability was >90%. Data not shown.

All figures are the mean values of 4 experiments and the standard error <5%

Fig 4.12a

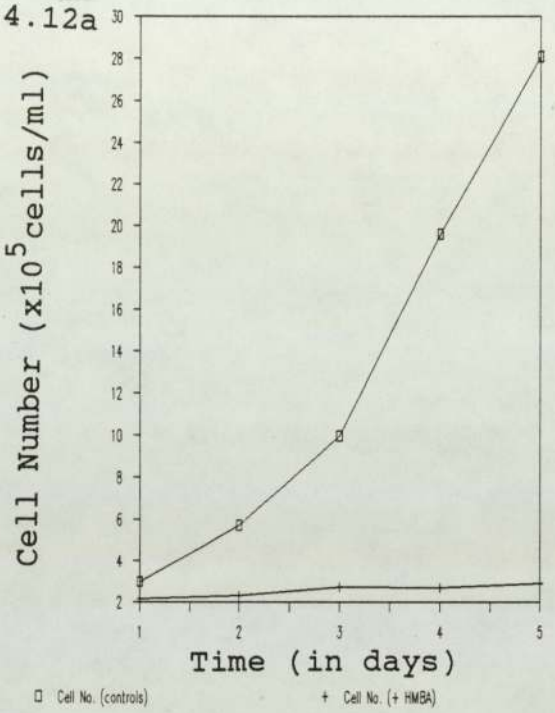
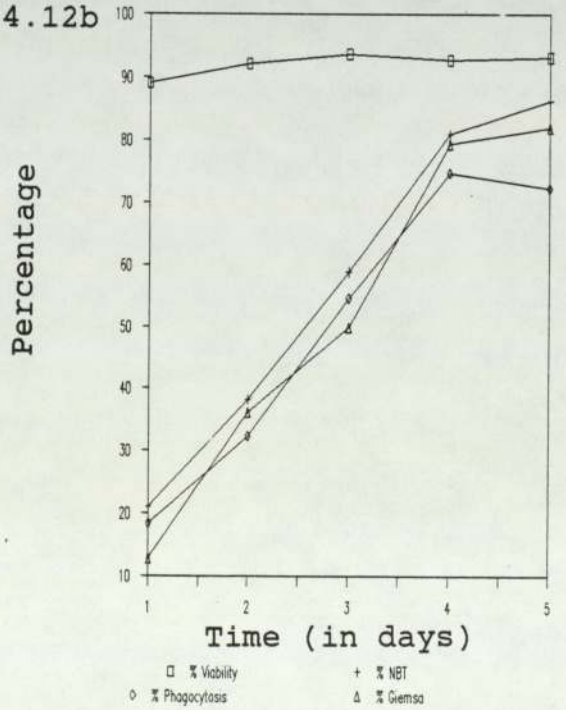


Fig 4.12b



4; and the cell viability was always in excess of 90% (see section 4.1a and 4.1b for discussion on commitment and NSE activity).

4.2. Commitment of the cells to differentiation with NMF, NEF, TMU, DBF, TMBF and HMBA

Whilst it took at least 2 days of incubation with the various inducers of granulocytic differentiation to induce detectable increases in the markers of cell differentiation, it is possble that events which commit the cells to differentiate occur much faster and, that once committed, it then takes the cells several days to fully express the markers. Experiments were performed to determine the time that the cells needed to be incubated with the optimum *in vitro* differentiating concentration of each of the inducers so as to commit them to differentiate, even in the subsequent absence of the inducer (Figs.4.13-4.18).

Differentiation was assessed 72 hours after the start of the experiment and it was shown that no significant commitment occurred before 12 hours incubation with any of the inducers. From 24 hours onwards, there was an almost linear increase in the percentage of cells committed to differentiate which reached a maximum at 48 hours. These

observations were in contrast to the commitment period that was required with inducers of monocytic differentiation where commitment occurred much more rapidly and the cells did not need to undergo one cell division cycle (see section 5).

The apparent lag phase was interesting as these drugs would have entered the cells almost instantaneously, and so it was unlikely that the 24 hour period represented the time required to increase the intracellular levels of the inducers to a threshold level above which differentiation was induced. It had previously been determined that HL-60 cells required incubation for a minimum of 24 hours before commitment occurred in response to retinoic acid and DMSO (Fibach et al, 1982., Tarella et al, 1982., Yen et al, 1985., Yen,1985), whereas commitment occurred more rapidly in response to TPA and RPA (Fibach et al, 1982). Yen & Albright (1984) suggested that HL-60 cells required at least 24 hours incubation with retinoic acid before commitment occurred because retinoic acid uptake was limited to S phase of the cells cycle. Thus, the uptake of the inducers was not responsible for the 24 hour precommitment period which occurred during NMF, NEF, TMU, DBF, TMBF and HMBA induced differentiation because, as previously mentioned, these inducers would have reached the cells almost instantaneously.

As the 24 hour time period approximately represented one

FIG.4.13.Results of the assay to determine the length of time required to commit HL-60 cells to differentiate using 170mM NMF.

Fig 4.13a - Cell Numbers measured 72 hours from the start of the experiment.
Fig 4.13b - Assays of differentiation.

All figures are the mean values of 3 experiments and the standard error <5%

Fig 4.13a

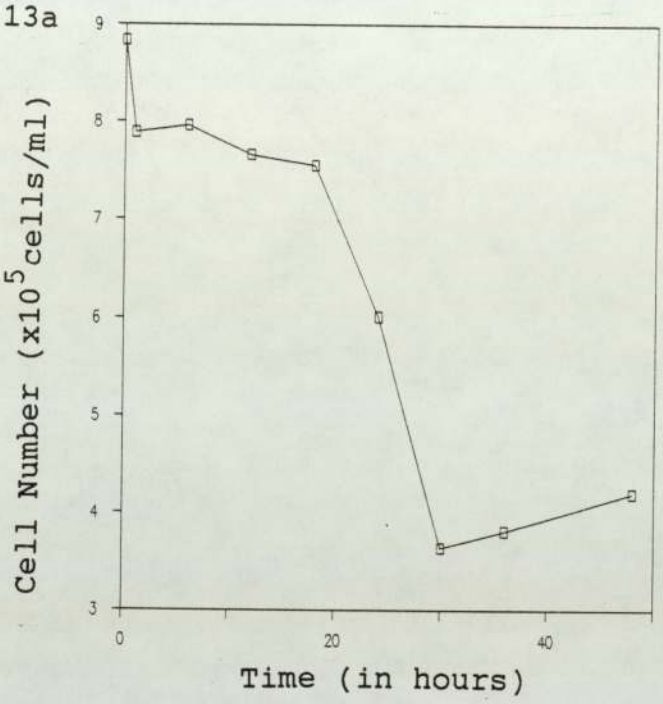


Fig 4.13b

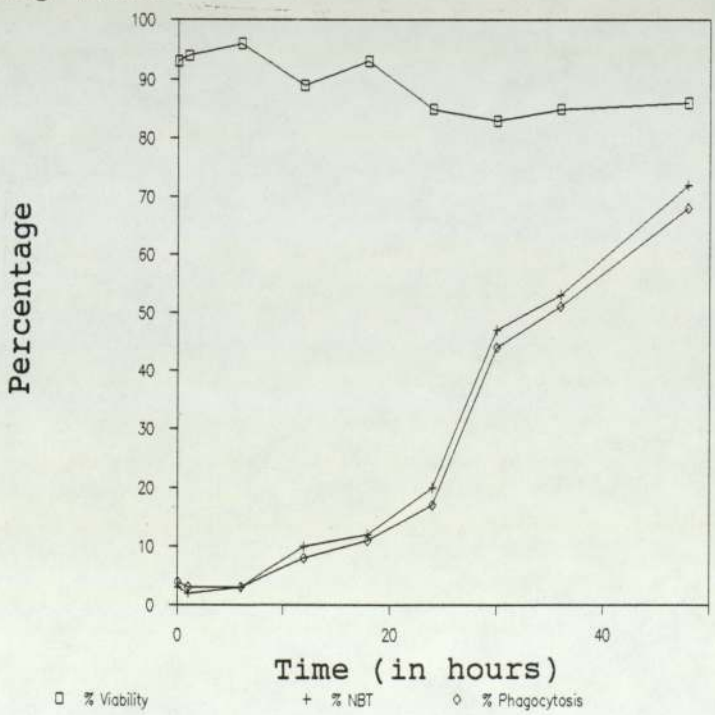


FIG.4.14.Results of the assay to determine the length of time required to commit HL-60 cells to differentiate using 80mM NEF.

Fig 4.14a - Cell Numbers measured 72 hours from the start of the experiment.
Fig 4.14b - Assays of differentiation.

All figures are the mean values of 3 experiments and the standard error <5%

Fig 4.14a

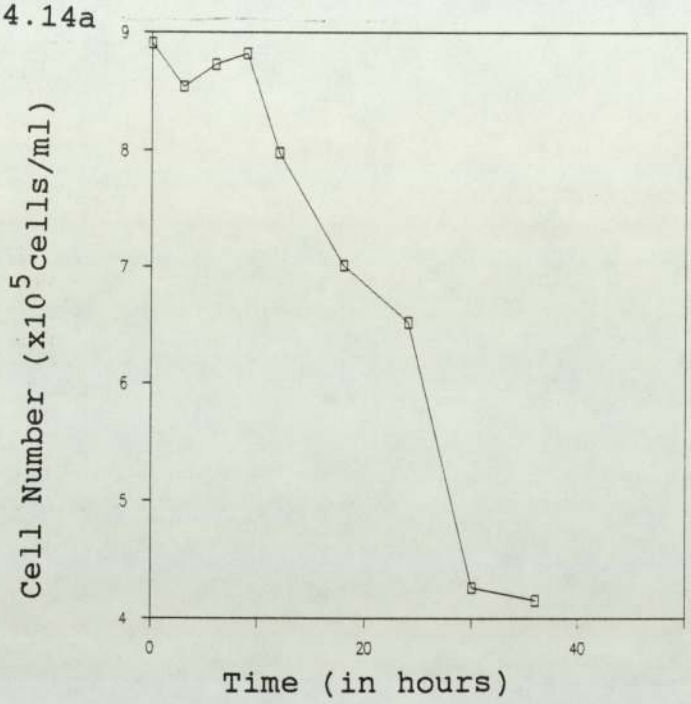


Fig 4.14b

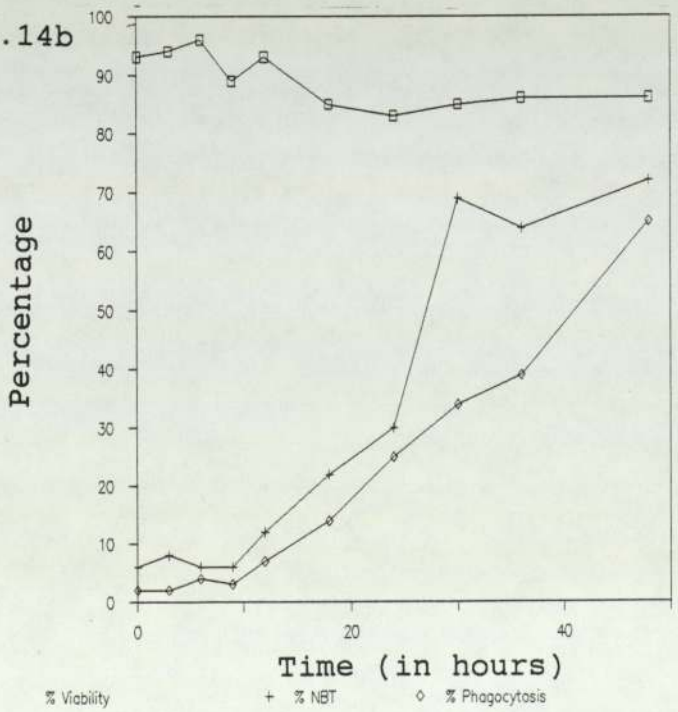


FIG.4.15. Results of the assay to determine the length of time required to commit HL-60 cells to differentiate using 8.5mM TMU.

Fig 4.15a - Cell Numbers measured 72 hours from the start of the experiment.
Fig 4.15b - Assays of differentiation

All figures are the mean values of 3 experiments and the standard error <5%

Fig 4.15a

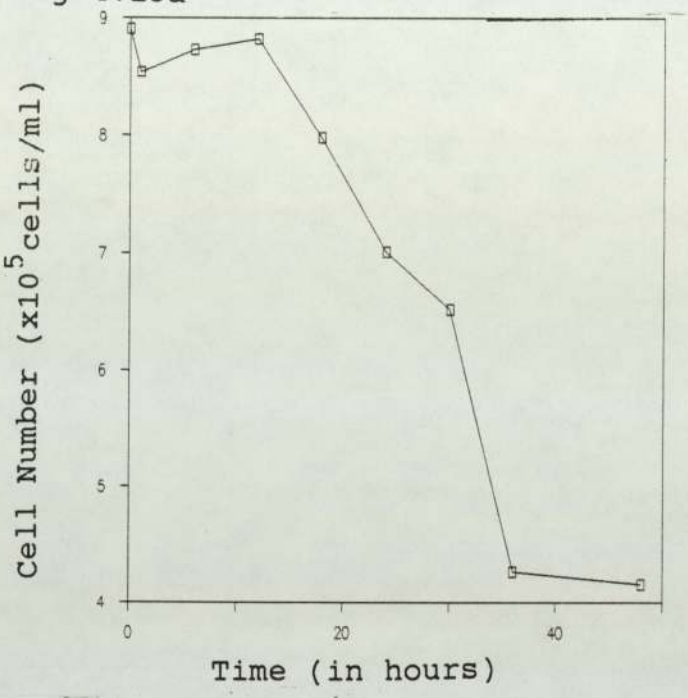


Fig 4.15b

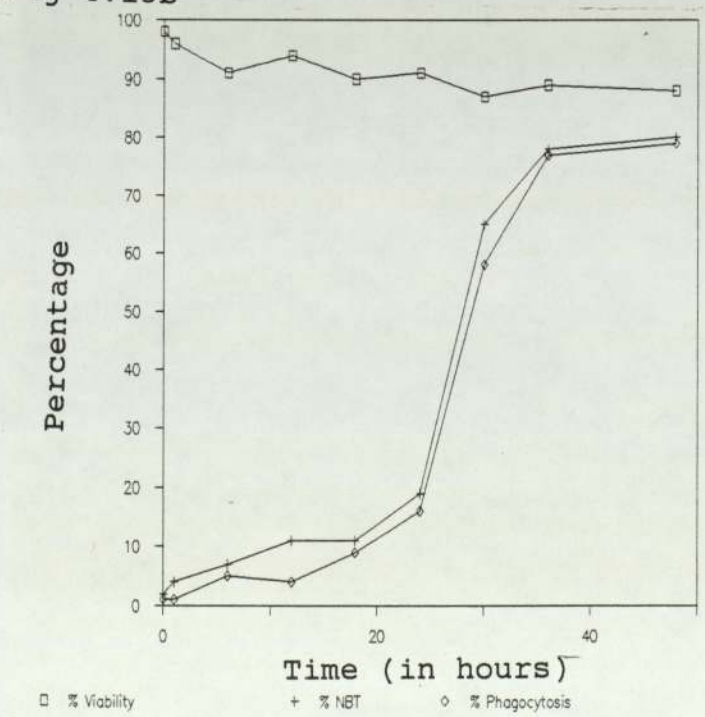


FIG.4.16.Results of the assay to determine the length of time required to commit HL-60 cells to differentiate using 1mM DBF.

Fig 4.16a - Cell Numbers measured 72 hours from the start of the experiment
Fig 4.16b - Assays of differentiation.

All figures are the mean values of 3 experiments and the standard error <5%

Fig 4.16a

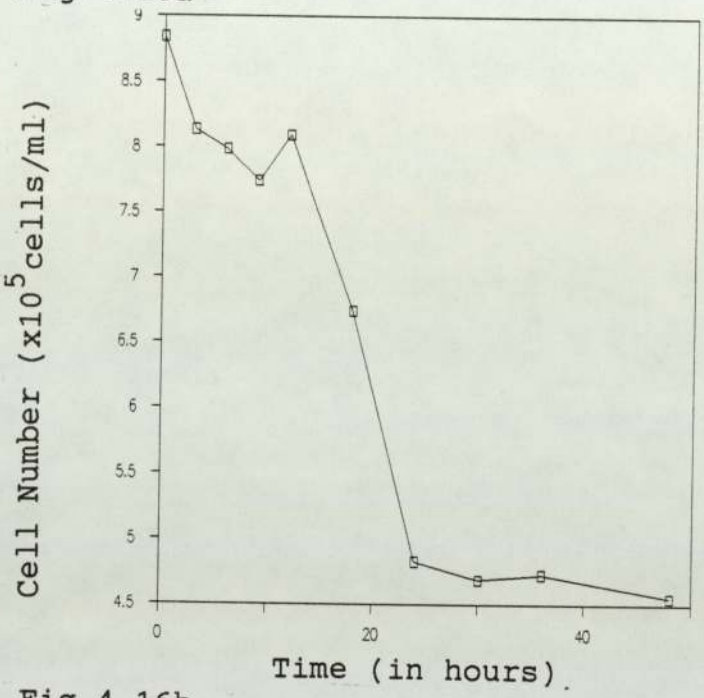


Fig 4.16b

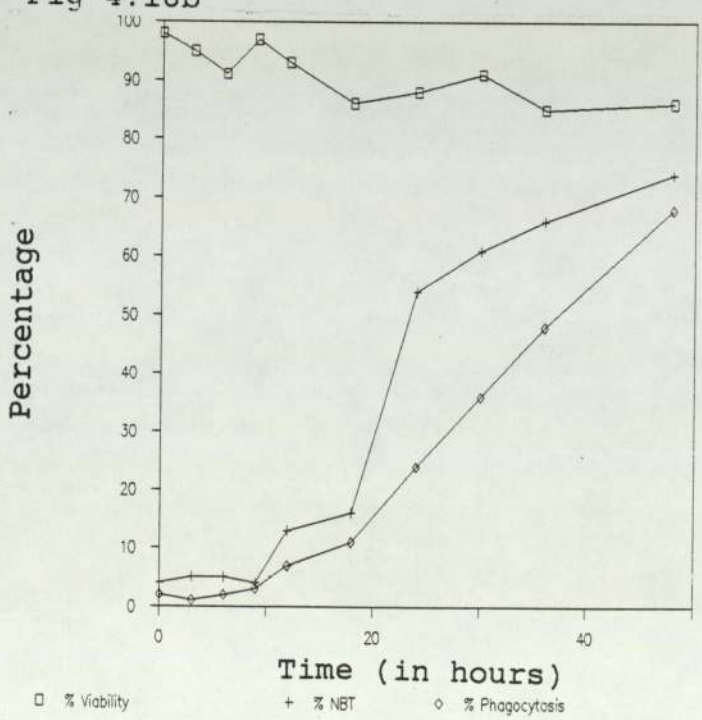


FIG.4.17.Results of the assay to determine the length of time required to commit HL-60 cells to differentiate using 1mM TMBF.

Fig 4.17a - Cell Numbers measured 72 hours from the start of the experiment.
Fig 4.17b - Assays of differentiation.

All figures are the mean values of 3 experiments and the standard error <5%

Fig 4.17a

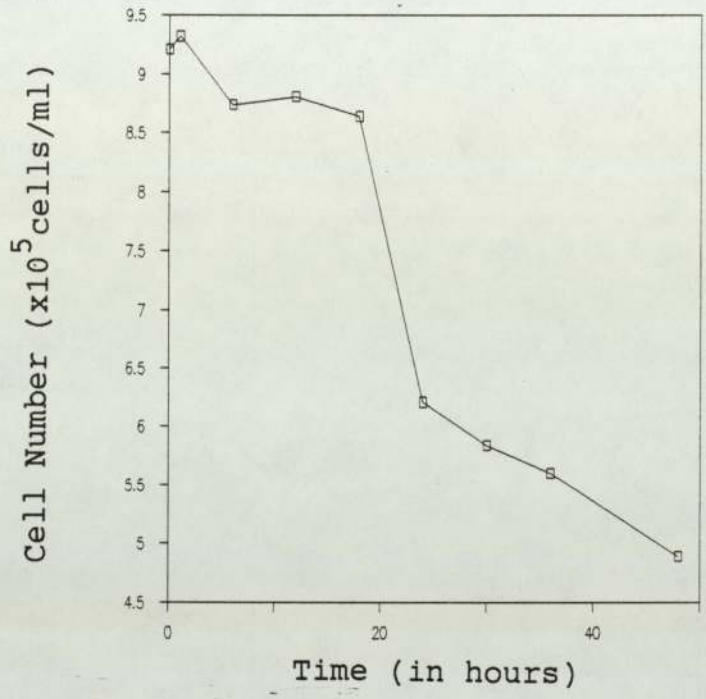


Fig 4.17b

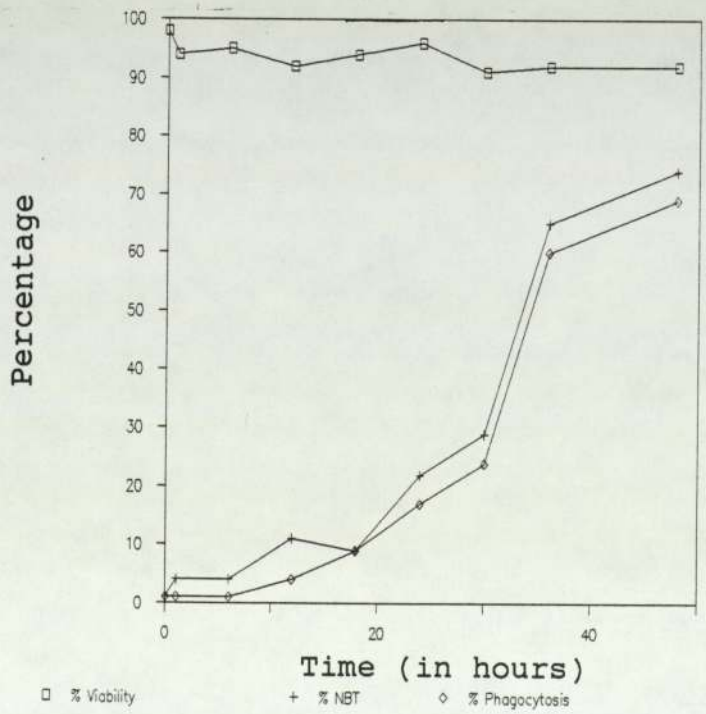


FIG.4.18. Results of the assay to determine the length of time required to commit HL-60 cells to differentiate using 2mM HMBA.

Fig 4.18a - Cell Numbers measured 72 hours from the start of the experiment.
Fig 4.18b - Assays of differentiation.

All figures are the mean values of 3 experiments and the standard error <5%.

Fig 4.18a

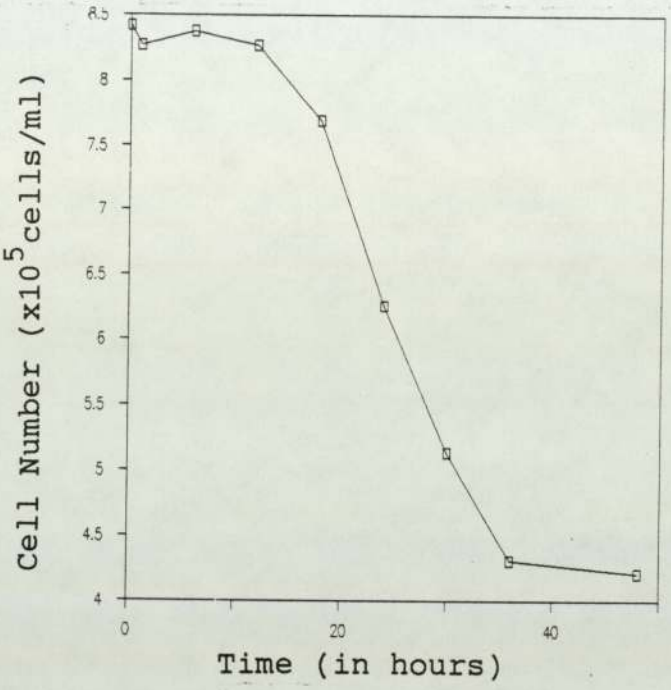
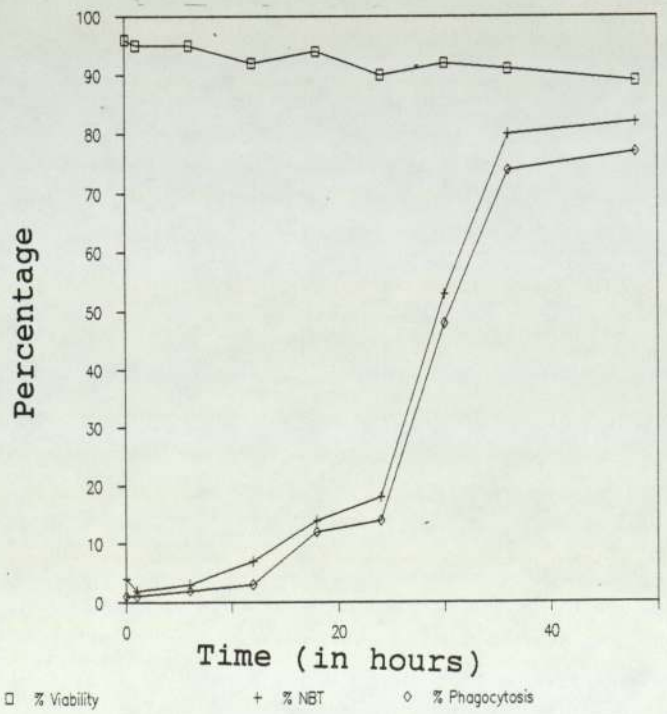


Fig 4.18b



cell cycle period, it was possible that commitment only occurred after the cells had undergone one full cell division cycle in the presence of the inducer. This suggested there was a restriction point for commitment in a certain phase of the cell cycle. This had been previously suggested by Boyd & Metcalf (1984), Yen & Albright (1984) and Studzinski et al (1985) as an explanation for the cell cycle phase-dependent induction of differentiation by retinoic acid and 1,25 dihydroxyvitamin D3. However, this hypothesis would not account for the evidence reported by Tarella et al (1982) which showed that the induction of differentiation was not cell cycle related.

This restriction point hypothesis is not consistent with the idea that the early steps in commitment are common to both granulocytic and macrophage lineages because commitment with TPA occurred from 4 hours onwards (as described in section 6.2) compared to 24 hours with the other inducers (Fibach et al, 1982., Yen et al, 1987). It is also in contrast with the findings reported by Tsiftsoglou et al (1985). They reported that DMSO could commit HL-60 cells, which had a cell doubling time of 34-36 hours, to differentiate after just 8 hours exposure to the inducer, further supporting the argument that commitment to differentiation is not linked to the cell cycle.

An alternative explanation for the 24 hour precommitment period required with inducers of differentiation such as NMF and NEF, is that the inducers caused a gradual accumulation of cellular damage which reached a critical level after 24 hours which initiated a stress response which resulted in commitment of the cells to differentiate. Agents such as TPA could induce the putative damage more rapidly than NMF or NEF, resulting in more rapid commitment of the cells to differentiate.

Langdon & Hickman (1987) suggested that a crucial characteristic of potential differentiating agents is that they must be present at a sub-toxic concentration for the induction of differentiation of HL-60 cells, further supporting the idea that commitment and differentiation of HL-60 cells is a response to stress.

4.3.a. Characterisation of the growth of HL-60 cells in diffusion chambers in vitro.

Culture of HL-60 cells in diffusion chambers in vitro is shown in figure 4.19. Cells both with and without serum were cultured in RPMI alone or 10% FCS/RPMI in diffusion chambers which were constructed using 0.45, 0.22 and 0.1μm filter membranes. The cells in the

Fig 4.19. Growth of HL-60 Cells in Diffusion Chambers in vitro.

Fig 4.19a - Growth of HL-60 cells that were cultured in diffusion chambers constructed using 0.45μm filters.
Fig 4.19b - Viability of HL-60 cells that were cultured in diffusion chambers constructed using 0.45μm filters.
Fig 4.19c - Growth of HL-60 cells that were cultured in diffusion chambers constructed using 0.22μm filters.

Fig 4.19a

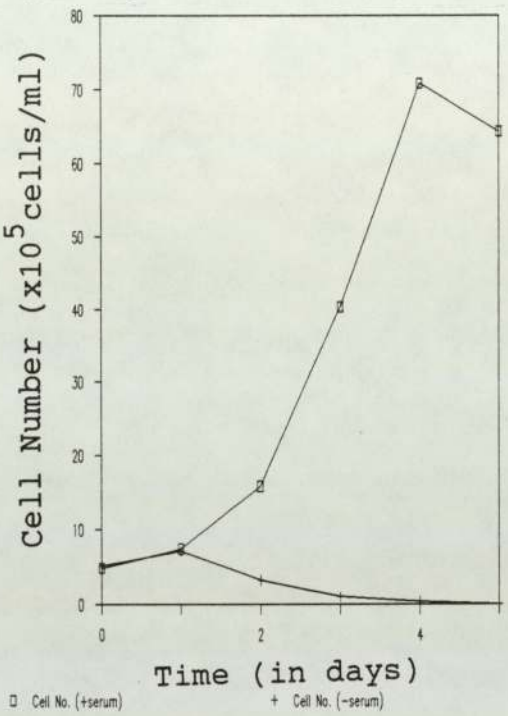


Fig 4.19b

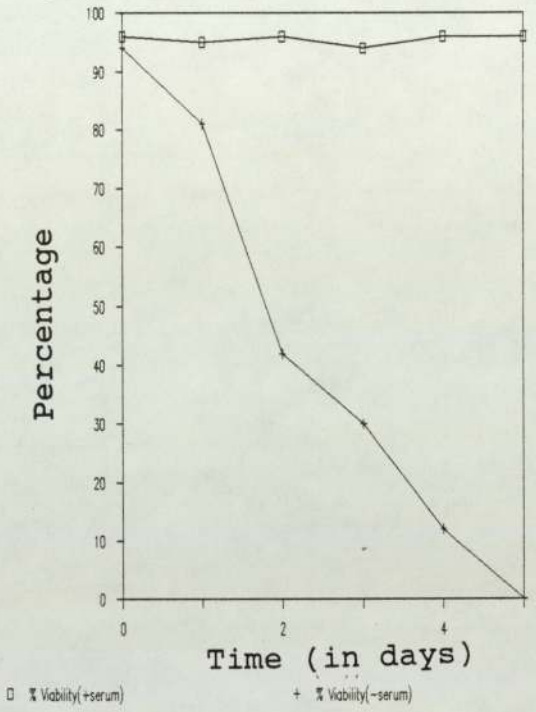


Fig 4.19c

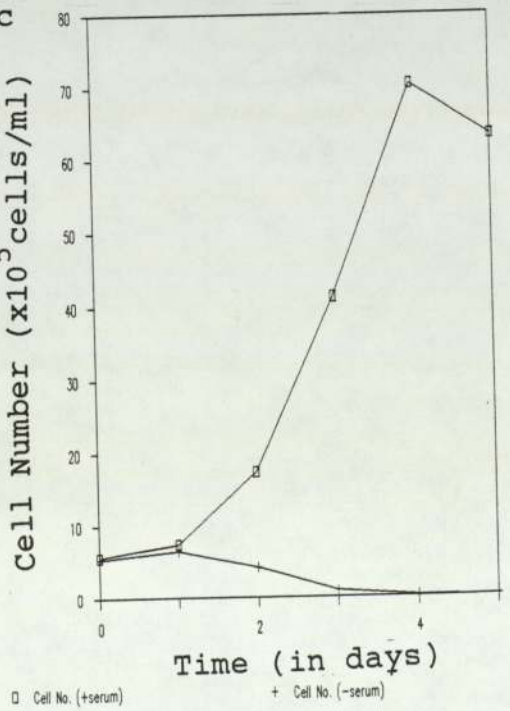


Fig 4.19. Growth of HL-60 Cells in Diffusion chambers in vitro.

Fig 4.19d - Viability of HL-60 cells that were cultured in diffusion chambers constructed using 0.22µm filters.
Fig 4.19e - Growth of HL-60 cells in diffusion chambers constructed using 0.1µm filters.
Fig 4.19f - Viability of HL-60 cells in diffusion chambers constructed using 0.1µm filters.

Fig 4.19d

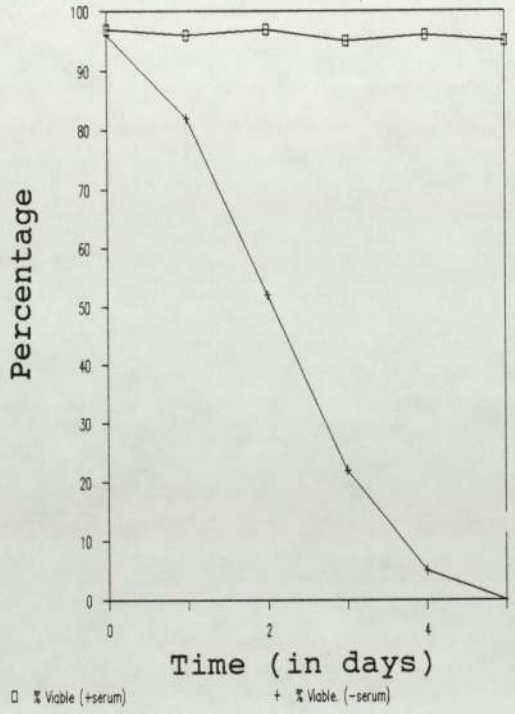


Fig 4.19e

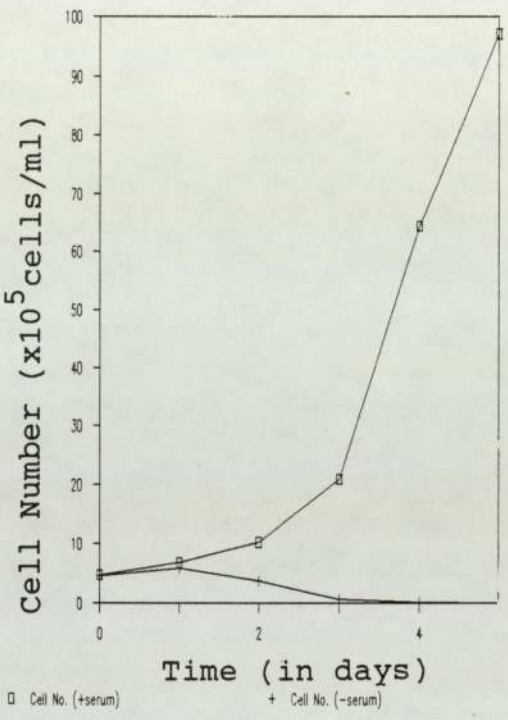
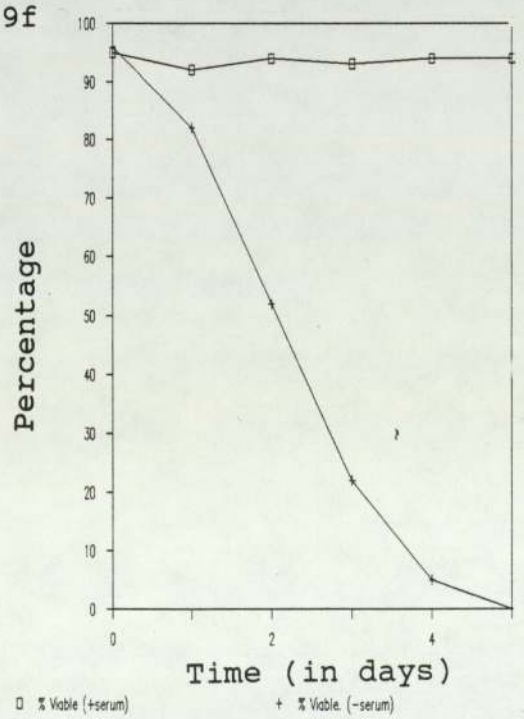


Fig 4.19f



serum-containing chambers exhibited similar growth characteristics within the chamber, as when they were growing freely in suspension with a cell population doubling time of approximately 28 hours. Cell growth reached a plateau on day 5 when the chambers were at their maximum capacity. There was no significant difference between the cell growth in chambers which had been constructed from the various pore sized filter membranes. However, in the serum-free chambers, the cells did not proliferate but decreased in number, and by day 4 only 10% of the remaining cells were viable.

4.4. Characterisation of the in vivo model of Cell Differentiation

Implantation of the chambers into the mice resulted in a weight loss of 2-3g during the first 4 days *in vivo* (table 4.2). It was thought initially that this was possibly due to the physical presence of the diffusion chamber within the abdominal cavity. However, when a "sham" implantation was performed with the mice undergoing the anaesthetic and surgery but without the implantation of the diffusion chamber, the mice lost a comparable amount of weight suggesting the trauma of the implantation technique strongly contributed to the observed weight loss. Each chamber was filled with 150µl of cell suspension and, on

Table 4.2. Weight Loss that occured upon implantation of the chamber.

Treatment	DAY 0	1	2	3	4
No chamber	21.3	21.2	21.4	21.4	21.4
Chamber	21.3	19.3	19.0	19.0	19.3
~Sham~	22.5	21.5	21.1	20.8	21.0

All figures are the mean weights of 3 mice. The weight losses of the control mice, mice that either contained a chamber or mice that had undergone the anaesthetic and incision but without the implantation of a chamber were recorded over a 4 day time period.

removal from the animal, the volume was always made up to 150µl to enable comparisons to be made between the chambers. Most of the chambers had a capacity of 150-250µl but there was much variabilty between the chamber volumes.

Growth kinetics of HL-60 cells within diffusion chambers, constructed using 0.45µm filters and implanted into BDF1 mice is shown in fig 4.20. The chambers were implanted within the peritoneal cavity of the mice using a central incision site (fig 4.21). The cells grew rapidly within the diffusion chambers with a cell doubling time of approximately 6/8 hours. The cell number reached a plateau after 2/3 days in culture in vivo and thereafter the cell number declined. The plateau cell number was reached at approximately the full capacity of the chamber; the volume of the chamber was calculated to be 1.57×10^{11} μm^3 and the volume of a cell was calculated to be 1436 μm^3 (using an average cell diameter of 14µm). There was no marked difference in cell growth in those chambers which contained cells both with and without 10% FCS and there was in excess of 90% viability in all the chambers. The percentage of spontaneous differentiation, as measured by the assay of the reduction of NBT, was higher in those chambers which contained the 10% FCS reaching 15-16% on days 6 and 7 in culture whereas the serum-free chambers exhibited only minimal spontaneous differentiation. The NSE assay was negative for all cells from the chambers which

FIG.4.20. Growth of HL-60 Cells in Diffusion Chambers In Vivo.

Figs 4.20a and 4.20b.

Diffusion chambers, which contained HL-60 cells at a density of $5x10^4$ cells/ml, were implanted into BDF1 mice using a central incision site. The chambers were removed from the mice at various time periods and incubated in 0.025% pronase solution to lyse the plasma clot. The filter membranes were broken open and the contents removed and counted.

All figures are the mean values of replicate chambers.

Fig 4.20a

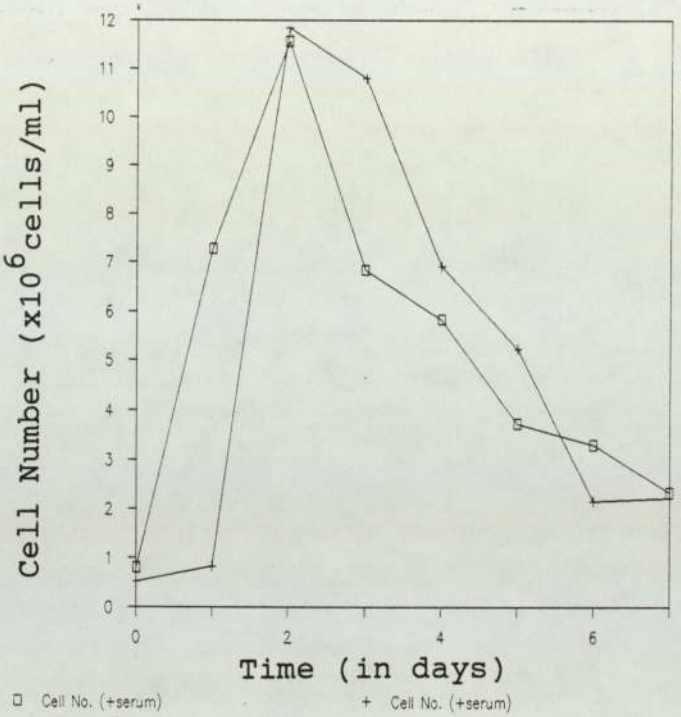


Fig 4.20b

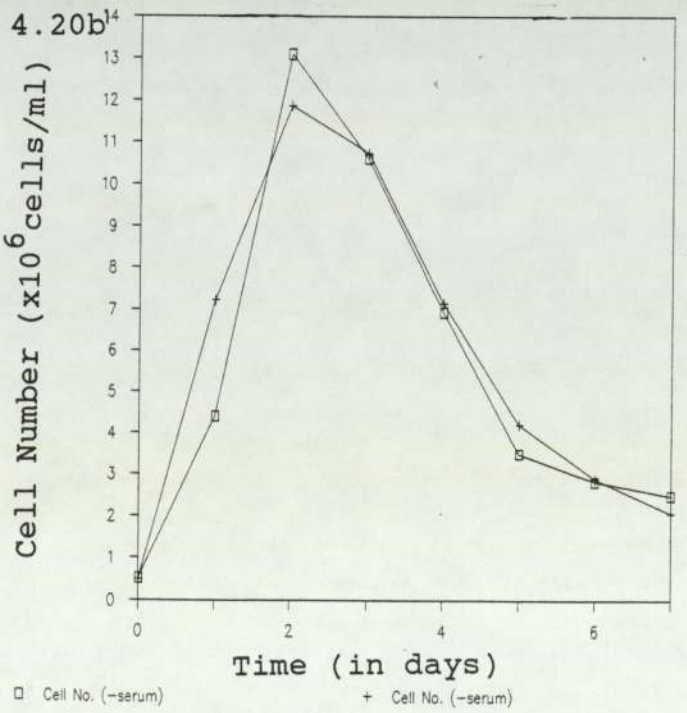


FIG.4.21. Diagram of the 2 Chamber Implantation Sites.

Fig 4.21a Centrally positioned implantation site.

Fig 4.21b Lateral implantation site.

Fig. 4.21a

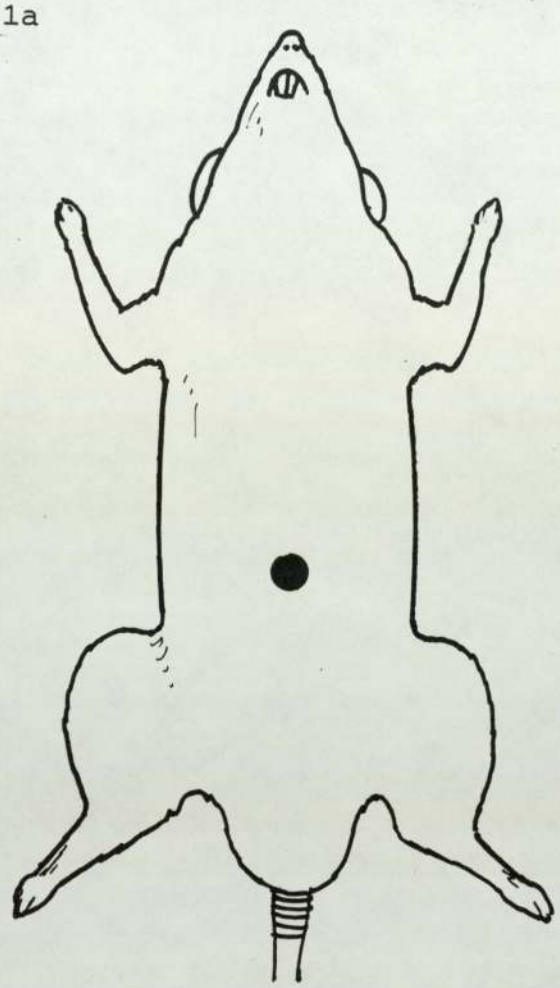
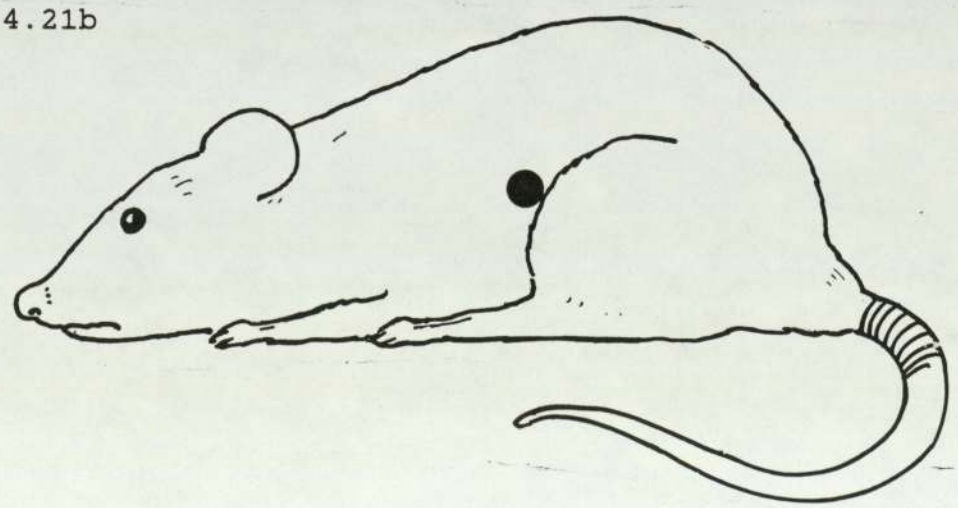


Fig. 4.21b



suggested that the cells that had differentiated had done so to granulocytes and not to monocyte-macrophage cells.

As mentioned in section 1.6.7., Anderson et al (1983) have cultured L1210 cells in diffusion chambers in vivo. They reported that the cells grew immediately and rapidly upon implantation of the chamber and reached a plateau after 4/5 days in culture. Similarly, Schwartz et al (1983) cultured HL-60 cells in agar in diffusion chambers which were implanted into mice and, likewise, they reported that the cells grew immediately and rapidly upon implantation of the chamber. The reason for such rapid growth rate is unclear. It could possibly have been due to the location of the incision site. A central incision site - which was stitched and clamped up - would always have been in contact with the bars of the cage as the animal moved about, resulting in a permanent irritation to the wound site. Such constant irritation would have prolonged the healing process with inflammatory, growth and repair factors being constantly present in the vicinity of the wound. The chamber would, therefore, always be close to such locally produced factors - even if it were placed to either side of the peritoneal cavity - and such factors might have provided a growth-stimulating environment for the HL-60 cells within the diffusion chambers.

Anderson et al (1983) had implanted the diffusion chambers into mice via a "midline abdominal incision site" which

had been sealed using surgical clips. This was analogous to the situation in the mice used in this work - with immediate and rapid growth of the implanted cells occurring in both cases. To allow for such a rapid growth rate, Anderson et al (1983) had only injected 100µl of the cell suspension into each chamber. This then provided 4/5 days of in vivo culture, when the cells were in log phase growth and before they reached a plateau phase of growth, which enabled them to study the effect of cyclophoshamide on the growth rate of the implanted cells. It might, therefore, have been possible to obtain such growth characteristics with HL-60 cells if the chambers had been filled initially with 100µl of cell suspension instead of 150µl.

Lotem & Sachs (1983) have shown that HL-60 leukaemia cells and mouse $MGI^{+}D^{+}$ myeloid leukaemic cells spontaneously differentiated to granulocytes when cultured in diffusion chambers implanted in the peritoneal cavity of SJL/J mice. This was demonstrated to be due to the presence of a xenogeneic but not a syngeneic or allogeneic serum within the chamber. This differentiation was associated with an increased intraperitoneal accumulation of inflammatory cells, particularly eosinophils. The response was markedly decreased in congenitally athymic and neonatally thymectomised mice and therefore it was suggested that spontaneous in vivo differentiation required the presence of T lymphocytes. Their most recent work has shown that

spontaneous in vivo differentiation could be induced by antigen-specific helper T cells and that this was associated with the ability of these cells to produce myeloid cell differentiating factor, MGI-2 (as described in section 1.3.2.). The antigen-specific helper T cells can accumulate at the site that contains the antigen (ie FCS) and thus can "control" in vivo differentiation (Lotem & Sachs, 1986). Further studies (Lotem & Sachs, 1983) demonstrated that injection of the inflammation-inducing compounds sodium caseinate, thioglycollate and bacterial lipopolysaccharide into mice induced the in vivo production of MGI-1 and MGI-2 - the growth and differentiation-inducing proteins - and the subsequent differentiation of the implanted cells. However, the differentiation of HL-60 cells in vivo appeared to be due to compound(s) other than MGI-2 as mouse MGI-2 did not induce the differentiation of these cells in vitro (Lotem & Sachs, 1979). In addition, human MGI-2 or compounds that induced the production of human MGI-2 in these leukaemic cells, induced HL-60 cells to differentiate to macrophages (Lotem & Sachs, 1979, 1980). Their results indicated that the in vivo conditions regulated the production of some growth factor(s) other than MGI-2 that induces the differentiation of HL-60 cells to granulocytes.

It was possible, therefore, that the rapid growth rate of the cells within the chambers was due to an accumulation

of growth and repair factor(s) produced during an inflammatory response. The HL-60 cells, even in those chambers that contained the xenogeneic serum, FCS, had not spontaneously terminally differentiated to granulocytes, which was in contrast to the work reported by Lotem and Sachs (1981). However, Lotem and Sachs had cultured the HL-60 cells *in vivo* for 10 days before assaying them for terminal differentiation, whereas the chambers in these experiments had only been cultured *in vivo* for 7 days, and it was possible, but unlikely, that the cells might have been committed to differentiation but were not yet expressing the markers of terminal cell differentiation.

The rapid and immediate growth of HL-60 cells in diffusion chambers that was observed by Schwartz *et al* (1983) could also have been due to stimulation by the products of an inflammatory reaction, although there was no mention of the site of the incision. In addition, their experiments had utilised a diffusion chamber that contained agar, and it has been suggested by Gordon and Blackett (1976) that the agar system substantially altered the response of the implanted cells to drugs and growth factors, possibly by altering the clearance rate of such compounds from the chamber (see section 1.6.7. for discussion on the diffusion chamber that utilises agar).

Similarly, Chikkappa *et al* (1980) reported that implantation of diffusion chambers, which contained human

bone marrow cells and cells from a patient with chronic myeloid leukaemia, into Hale-Stoner Brookhaven mice resulted in an increase in cell numbers after 2 hours in in vivo culture. The cell numbers then increased to a maximum at 48 hours and then decreased to a minimum at 240 hours. It was further demonstrated that the granulocytic maturation sequence occurred in the diffusion chamber in an orderly fashion, as it does in man. However the rate of maturation of the cells from one stage to the next was faster in the diffusion chamber than in man as was demonstrated in simultaneous studies in patients and their cells in diffusion chambers. This fact also demonstrated that the cells of patients with chronic myeloid leukaemia were still responsive to the factor(s) influencing cell growth and differentiation in the culture system and that the mouse was providing factor(s) that accelerated maturation. No attempts were made to isolate and identify these factors.

There was, again, no mention of the site of the incision made in the mice by Chikkappa et al (1980) and their data, with respect to the differentiation of the leukaemic cells, was in contrast to the work presented here although the growth characteristics of the cells were similar. The diffusion chambers were, however, implanted into mice that had previously been irradiated and they were then transferred into newly irradiated mice every 7 days, with the total in vivo culture period being 18 days. Both of

these factors might have contributed to the induction of differentiation of the leukaemic cells as irradiation of the host animal has been shown to result in increased granulopoesis within a diffusion chamber, as outlined in section 1.6.7. Irradiation of the host animal would result in an altered in vivo environment which could have affected the chamber contents, and an 18 day time period should have been sufficient for the cells to express markers of terminal cell differentiation.

The growth characteristics of the implanted HL-60 cells were not considered representative of the growth of such cells in vitro, which had a cell doubling time of 28 hours compared with 6/8 hours within the chambers. In addition, HL-60 cells generally have to be able to divide at least once before they will differentiate to granulocytes in response to exposure to various inducers (as described in section 1.5) and this model only provided 2 days of such growth during which time the potential differentiating agents could be administered.

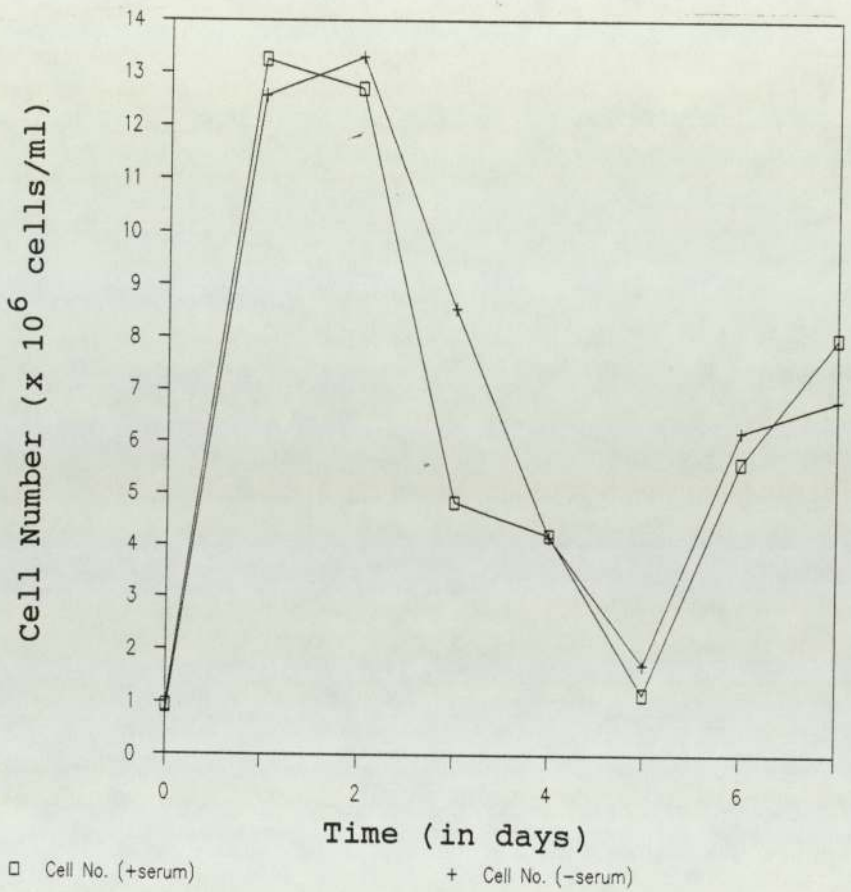
No differences in HL-60 cell growth in diffusion chambers were observed when the chambers were implanted into either BDF_1 or CBA mice (figs 4.20., 4.22). This was in accordance with observations by Benestad & Reikvam (1975) who demonstrated that there was no difference in the growth of human bone marrow cells in diffusion chambers implanted in C3H x DBA/2 or NMRI mice - their results were

FIG.4.22. Growth of HL-60 Cells in Diffusion Chambers In Vivo.

Diffusion chambers, which contained 5×10^5 HL-60 cells at an initial density of 1x10^5 cells/ml, were implanted into CBA/Ca mice using a central incision site. The chambers were removed from the mice at various time intervals and incubated in 0.025% pronase solution. The filter membranes of the chamber were broken open and the contents removed and counted.

All figures are the mean values of replicate chambers.

Fig 4.22



not influenced by sex or strain differences between the hosts.

In contrast, when the chambers were implanted ip through a lateral incision site, (fig 4.21b) with the chamber being placed distal to the wound site, an initial growth lag of 2-3 days was observed before the cells resumed logarithmic phase of growth (fig 4.23). The cells then remained in logarithmic phase of growth for 4 days before reaching a plateau and thereafter the cell numbers declined. The plateau was again reached at approximately the full capacity of the chamber. The initial loss of HL-60 cells from the diffusion chamber during the first two days *in vivo* was marked (50-60%) Previous investigators have reported an even greater initial cell loss. In 1966, Nettesheim *et al* reported that, after only 4 hours of culture in diffusion chambers *in vivo*, mouse spleen cells showed a 90% reduction of the initial innoculum and this was not due to poor recovery techniques as the pronase-lysis treatment has been shown to release virtually all the cells from the chamber and free those cells attached to the filter surface. Likewise, Capalbo *et al* (1964) showed there was a 70-90% cell loss within the initial 24 hours of culture of spleen cells in diffusion chambers *in vivo*. Only tentative explanations for such a loss have been proposed. It has been mentioned that the cells have been mechanically stressed and that they have been exposed to a foreign environment. Some of

FIG.4.23. Growth of HL-60 Cells in Diffusion Chambers In Vivo.

Figs 4.23a and 4.23b.

Diffusion chambers were constructed using 0.45μm filters and were implanted into CBA/Ca mice using a lateral incision site. The initial cell density was 1 x 10^6 c/ml. All figures are the mean values of replicate chambers and the cell viability was >85%. The percentage of spontaneous differentiation was <10%.

Fig 4.23a

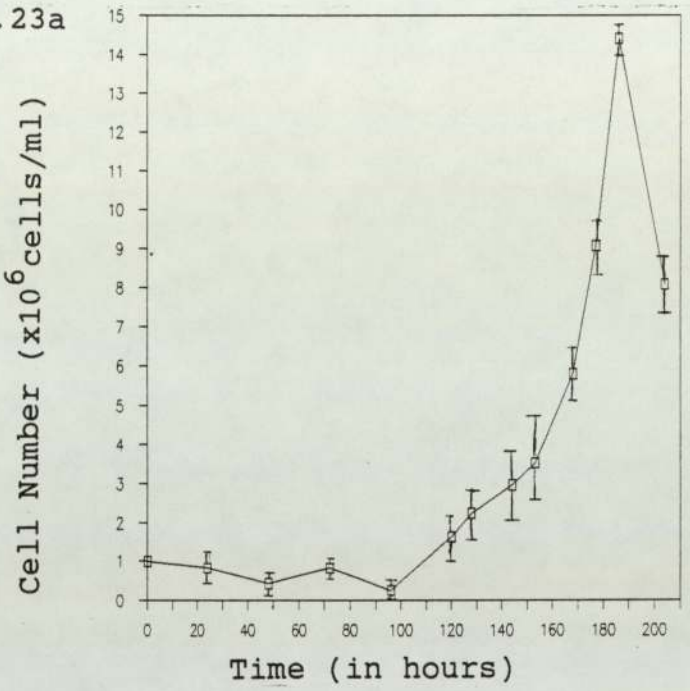
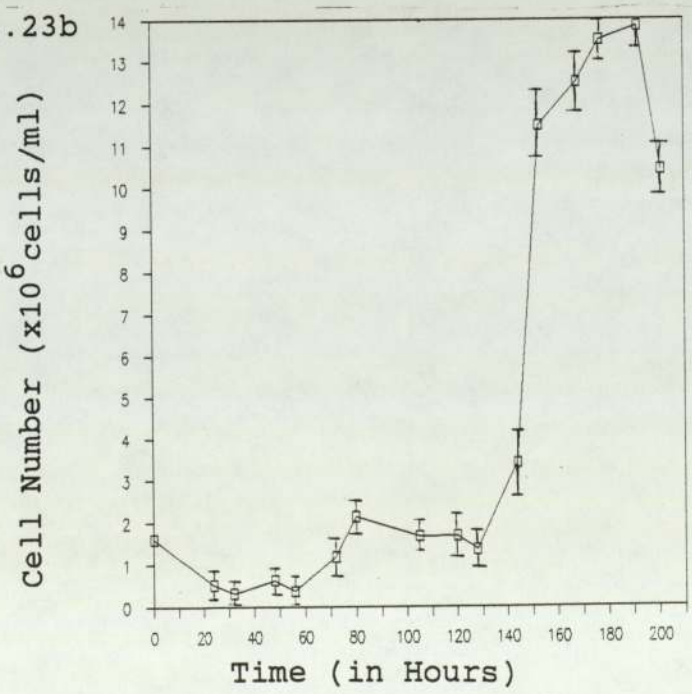


Fig 4.23b



the innoculated cells must naturally have a short normal life-span (Benestad, 1970). Other alternatives are that HL-60 cells are a naturally heterogenous population and that implanting these cells within a diffusion chamber *in vivo* selects the sub-population that is best suited to growth within such an *in vivo* environment. The remaining sub-populations of cells die in these new surroundings and the subsequent cell growth is the selected *in vivo* sub-population (Lotem & Sachs, 1980). Such a hypothesis would explain the lack of success obtained when the cells were removed from the implanted diffusion chamber and cloned in soft agar *in vitro*. The *in vivo* cells were not able to adapt to these *in vitro* growth conditions and hence did not clone (data not shown).

Similarly, Boyum *et al* (1972) have reported a marked lag phase of 8 days before human bone marrow cells resumed proliferation in diffusion chambers. There was an initial 70% cell loss after 1 day and thereafter the cell numbers varied between 40-60% of the innoculum for 7 days before there was an increased cell yield. There was no explanation offered for the initial cell loss.

A possible contribution to the initial cell loss was the Millipore membrane filters. Andrews (1970) demonstrated that the filters contained detergent which was toxic to the implanted cells and which resulted in poor culturing efficiency. However, this was unlikely to be a major

contributory factor as the work presented here utilised leukaemic cells which were shown to be virtually resistant to the toxic effects of the detergent-laden filter membranes. Similarly, the cement used to attach the filter membranes to the Lucite ring was shown to be toxic to some implanted cells lines (Page et al, 1986) (see section 1.6.2b.). Again, this was unlikely to be major contributory factor to the initial cell loss as the cement was not toxic to any of the tumour lines tested

Work by Langdon et al (1986) has demonstrated that HL-60 cells will only differentiate in vitro with NMF when they are able to undergo at least one cell division. Implantation of the chamber using a lateral incision site provided at least 4 days in vivo when the cells were growing logarithmically with a cell doubling time of approximately 28 hours. This was also preferable to the use of a central incision site because by the time the implanted cells had commenced log phase of growth, after 5 days in vivo, the mice had recovered from the trauma of the implantation procedure, as indicated by their increase in weight (Table 4.2). A lateral incision site was therefore used for all further experiments.

A major limitation with this system was that the variability of cell numbers within the chambers was approximately 20% when the cells were in the log phase of growth compared with approximately 5% during the initial 5

days of *in vivo* culture or when the cells were at a plateau phase of growth (fig 4.23). Thus, if the chambers were harvested during the log phase of growth this could have resulted in poor agreement between replicate chambers; the data might also have been difficult to interpret unless the sample number was large enough for statistical analysis. It was therefore decided to administer the potential differentiating agents for 4 consecutive days, whilst the cells were in log phase of growth, and to harvest and assess them 24 hours after the final administration of drugs; when the cells were at a plateau phase of growth. The drugs could not be administered to the mice during the initial days of *in vivo* culture as there were an insufficient number of cells in the chamber to perform accurate assessments of cell growth and differentiation; and also because of the extreme variation in cell numbers during the log phase of growth when the chambers would be harvested. The drugs could not be administered when the cells were at the plateau phase of cell growth because, as mentioned previously (section 4.1a), the cells have to be able to undergo at least one replication before they will become committed to terminal differentiation.

In addition, it was decided to harvest and assess the contents of the chambers 24 hours after the final administration of the drugs. This was not ideal and was a limitation of the system because there may have been cells

that were committed to differentiation but were not yet expressing the markers of terminal cell differentiation. As mentioned in section 4.1, the cells do not express markers of terminal differentiation until after 2 days incubation with the various agents. Alternatively, although harvesting the chambers at a later date might have shown an increased percentage of differentiation, there may have been outgrowth of the undifferentiated cells which would have affected the percentage of differentiation measured. In support of harvesting the chambers 24 hours after the final administration of the drugs, was the data shown in figs 4.2 and 4.4. The HL-60 cells treated with the various inducers of differentiation show a significant increase in the percentage of granulocytic differentiation after 2 days, and so any cells committed to differentiate after the 2nd/3rd administration of drugs <u>in vivo</u> may be considered to have expressed markers of differentiation when the chambers were harvested.

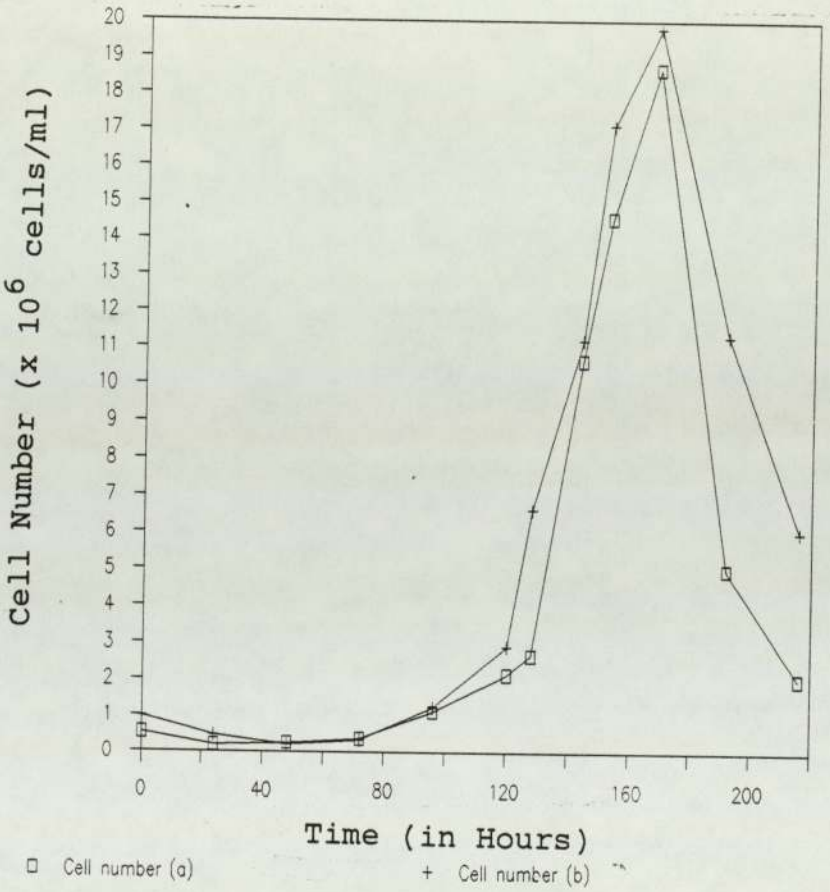
The growth of serum-free HL-60 cells in a diffusion chamber <u>in vivo</u> is shown in fig 4.24. The growth characteristics were very similar to those observed using HL-60 cells with serum. There was an initial lag phase of 48-72 hours, then log phase growth up to 153 hours with the cells reaching a plateau at 168 hours and the cell numbers declining thereafter. The percentage of spontaneous differentiation, as measured by the NBT assay,

FIG.4.24. Growth of Serum-free HL-60 Cells in Diffusion Chambers In Vivo.

Cells were washed free of FCS 3 times before being injected into the chamber and implanted into CBA/Ca mice. The diffusion chambers were constructed using 0.45μm filters and the initial cell density was 1 x 10^6 cells/ml. All figures are the mean values of replicate chambers and the cell viability was >85%
The percentage of spontaneous differentiation was <10% and the standard error was <5%.

Figures show the results of two experiments a and b.

Fig 4.24



showed only minimal granulocytic differentiation (<10%) and the viability was in excess of 85% throughout the experiment.

When the diffusion chambers were cultured *in vivo* over a 21 day period (fig 4.25), those chambers which contained serum-free HL-60 cells continued to show minimal differentiation - no more than 14% after 21 days. The chambers that contained HL-60 cells in 10% FCS showed increasing amounts of differentiation, as measured by the NBT assay, up to 60% after 21 days. The percentage of spontaneous differentiation started to increase after 11 days *in vivo* and there was no real change in cell numbers after this time. The viability of the cells in both experiments was in excess of 85%.

Similarly, Steele *et al* (1977) demonstrated that culture of human leukaemia bone marrow cells in diffusion chambers *in vivo* resulted in an initial rise in cell numbers, which was in contrast to culture of normal human bone marrow cells. These initially decreased in number during the first 7 days. Thereafter, the cell numbers fluctuated over a 28 day period with the cells neither increasing or decreasing significantly in number. Brevik *et al* (1971) have also reported that culture of mouse bone marrow cells in diffusion chambers *in vivo* resulted in an initial lag phase, followed by logarithmic phase of growth and a

FIG.4.25.The Growth Characteristics of HL-60 cells in Diffusion Chambers Implanted into CBA/Ca mice assessed over a 21 Day Time Period.

HL-60 cells both with and without 10% FCS were injected into chambers and implanted into CBA/Ca mice. The chambers were constructed using 0.45µm filters and were implanted using a lateral incision site. The growth characteristics of the cells were assessed over 21 days. The initial cell density was 1 x 10^6 cells/ml. All figures are the mean value of 2 chambers and the cell viability was >85%.

Fig 25a - Cell Viability and % NBT.
Fig 25b - Cell Numbers.

a - + 10% FCS
b - - 10% FCS

Fig 4.25a

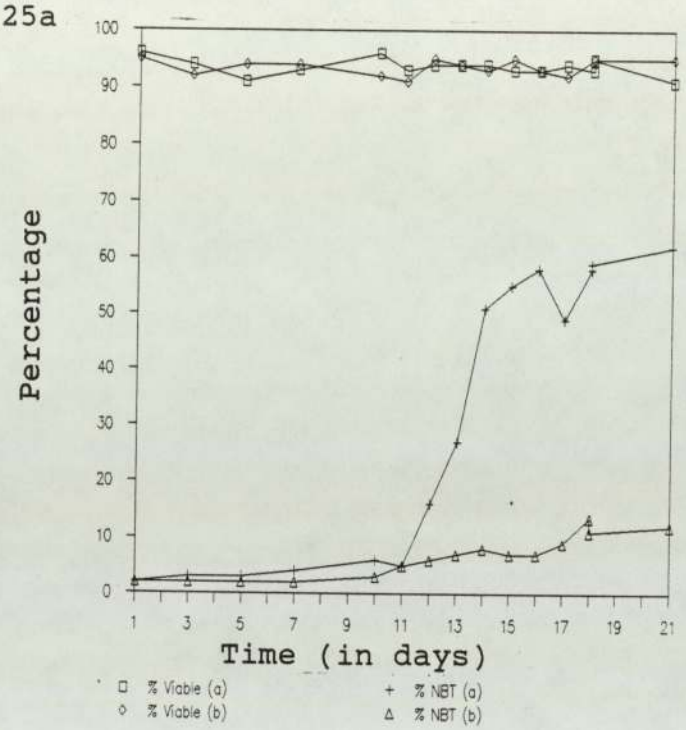
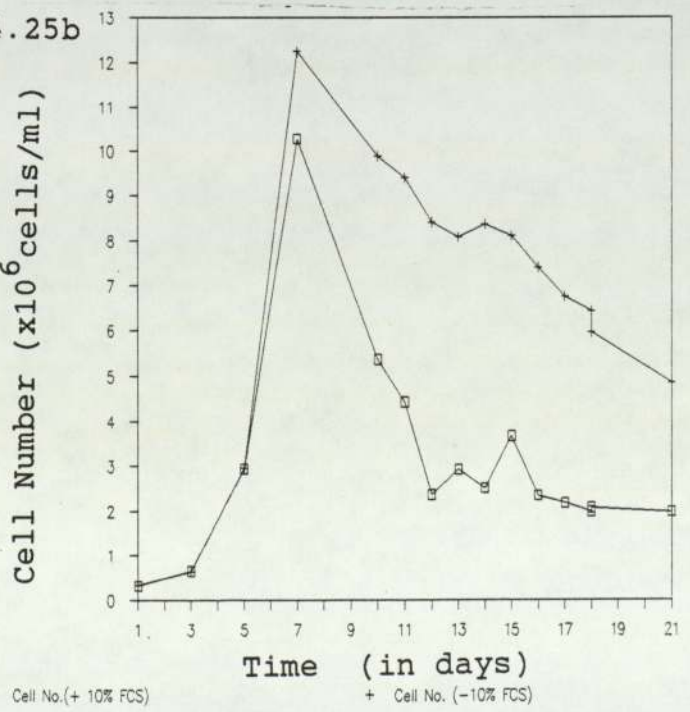


Fig 4.25b



plateau phase of growth after 7 days. The numbers then declined steadily and slowly up to 18 days with the cells never resuming log phase of growth.

Likewise the results presented here show that once the HL-60 cells have reached a plateau phase of growth within the diffusion chamber at 153 hours, the cell numbers declined to approximately $7x10^6$ cells/ml and thereafter fluctuated between $2-7x10^6$ cells/ml. The cells did not resume log phase of growth and reach a plateau phase again. An explanation for this phenomenon is that the cells have terminally differentiated and thus have lost their proliferative capacity. In the diffusion chamber that contained the serum, there was a significant increase in the percentage of terminal differentiation; however, there was no more than 14% differentiation measured in those chambers that contained the serum-free cells. Similarly, there was no *in vivo* differentiation of the leukaemic cells used by Steele *et al* (1977) who suggested that the defect in leukaemia is intrinsic to the leukaemia cell rather than primary a disturbance of the milieu into which the cell is placed. This was in contrast to the results reported by Chikkappa *et al* (1980) (see section 4.4) who showed significant spontaneous differentiation of CML cells in diffusion chambers *in vivo*.

It was possible that the culture period *in vivo* was not for a long enough time period (21/28 days); the cells

might have commenced growing again after this time period. Alternatively, it was possible that, due to coating of the external filter membranes with mouse cells and tissue, an adequate supply of nutrient and growth factors could not transverse the filter membrane to allow the cells to resume log phase of growth, but there were sufficient available to allow the steady-state growth of the cells implanted within the chamber.

The effects of altering the filter pore size on the growth characteristics of implanted HL-60 cells is shown in figs 4.23, 4.26, 4.27. Both the 0.45μm and 0.22μm filters supported good cell growth with a growth lag of 48-72 hours, a log phase and a plateau phase of growth at 150 hours. There was little difference in the yield of cells harvested from diffusion chambers constructed using these two filter pore sizes. In contrast, the 0.1μm filters supported only poor cell growth within the diffusion chamber, the cells did not appear to enter logarithmic phase of growth and the maximum cell number was reached at 1.4×10^7 cells/ml.

This maximum cell number was lower than that obtained when using 0.22 and 0.45μm filters. Work by Benestad & Reikvam (1975) has likewise demonstrated that the 0.22 and 0.45μm filters supported better mouse cell growth than the 0.1μm filters although no explanation was offered for this phenomenon. It was possible that the 0.1μm filters did

FIG.4.26. The Growth Characteristics of HL-60 cells in Diffusion Chambers, which were constructed using 0.22um Filters and implanted into CBA/Ca mice, assessed over various time periods.

The initial cell density was 1 x 10^6cells/ml and all figures are the mean value of 2 chambers which were constructed using 0.45µm filters and implanted using a lateral incision site. The cell viability was >85% and the percentage of spontaneous differentiation was <10%. The standard error was <5%.

Figures show the results of two experiments a and b.

Fig 4.26

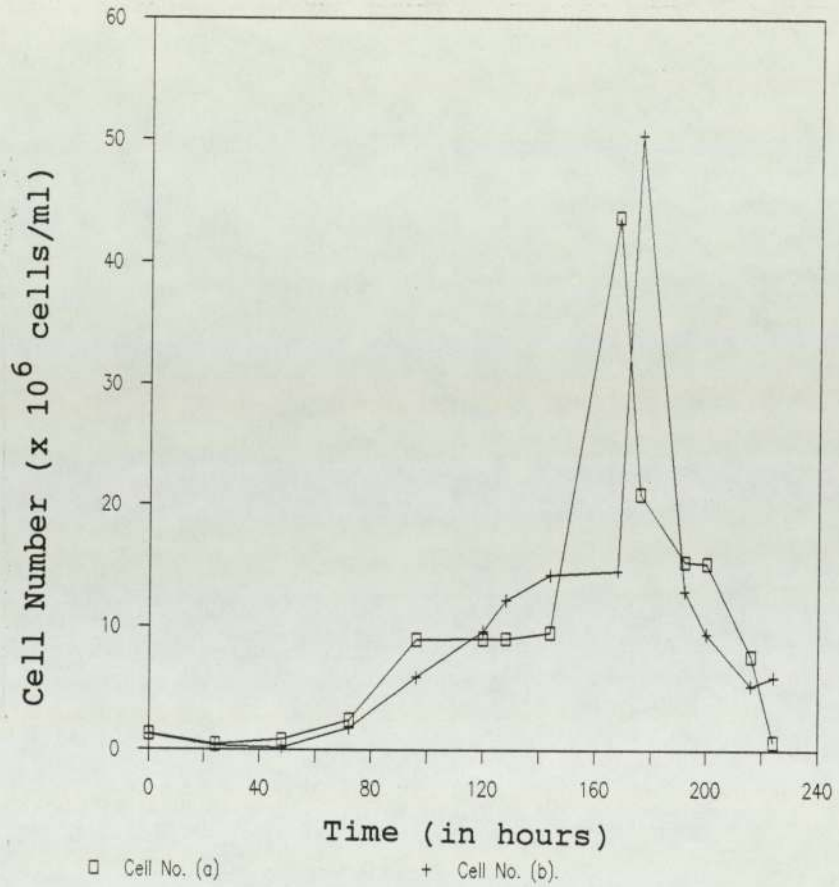
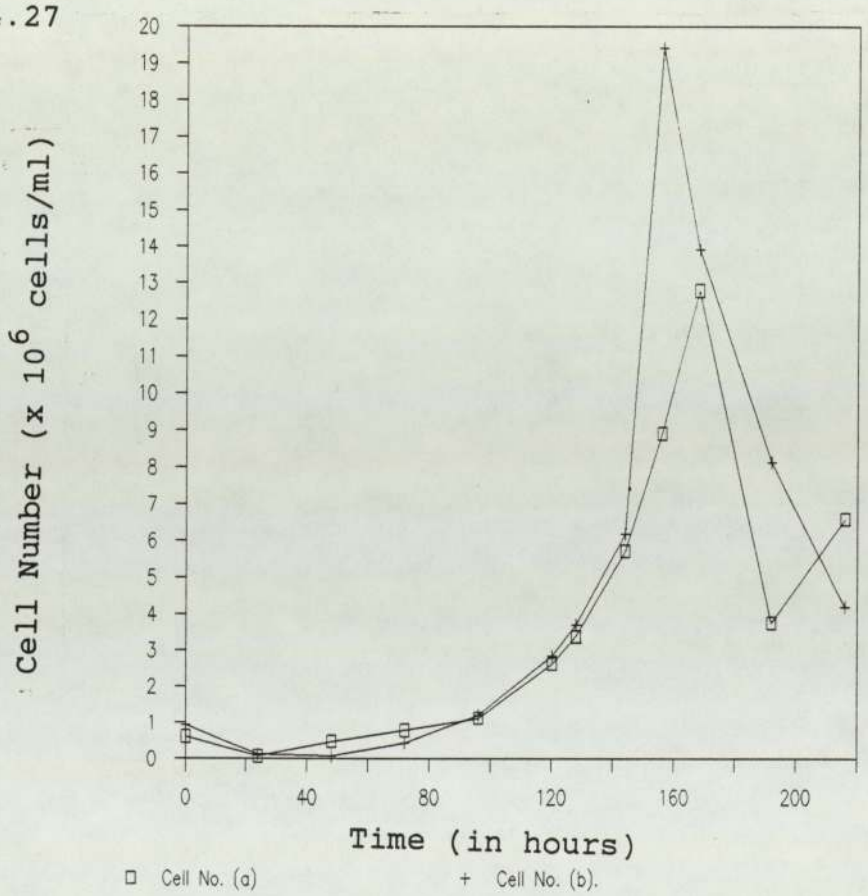


FIG.4.27. The Growth Characteristics of HL-60 cells in Diffusion Chambers, which were constructed using 0.10um Filters and implanted into CBA/Ca mice, assessed over various time periods.

The initial cell density was 1 x 10^6cells/ml and all figures are the mean value of 2 chambers. The chambers were constructed using 0.45µm filters and were implanted using a side incision site. Cell viability was >85% and the percentage of spontaneous differentiation was <10%. Standard error was <5%.

Figures show the results of two experiments a and b.

Fig 4.27



not permit an adequate supply of nutrients to enter the diffusion chamber and hence there was reduced cell growth when using these filters.

It was suggested that for normal bone marrow cells, the final plateau for cell number in diffusion chamber cultures varied as a function of the number of cells placed in culture, whereas for the leukaemic cell population the plateau of cell growth in diffusion chamber culture was not a function of the input number but represented the maximal capacity of the culture system irrespective of innoculum size (Miller et al, 1976).

For examination of mouse cells and tissues within diffusion chambers in vivo, it was suggested that 0.22μm filter membranes be used, but a filter pore size of 0.45μm could be used when human cells and tissues are cultured.(Carsten, 1984). However, other investigators appear to use both sizes irrespective of the cell type being cultured (Bab et al, 1986., Boyum et al, 1972). In further experiments a filter pore size of 0.45μm was used as it had been shown to support reproducible cell growth with good agreement in cell numbers between replicate chambers.

Varying the initial innoculum density injected into the chambers also affected the growth characteristics of the cells (figs 4.23, 4.28, 4.29). When the initial cell

FIG.4.28.The Growth Characteristics of HL-60 cells, at a high cell density of $5x10^6$ cells/ml, in diffusion chambers implanted into CBA/Ca mice assessed over various time periods.

All figures are the mean value of 2 chambers which were constructed using 0.45μm filters and were implanted using a lateral incision site. The cell viability was >85% and the percentage of spontaneous differentiation was <10%. Standard error was <10%.

Figures show the results of two experiments a and b.

Fig 4.28

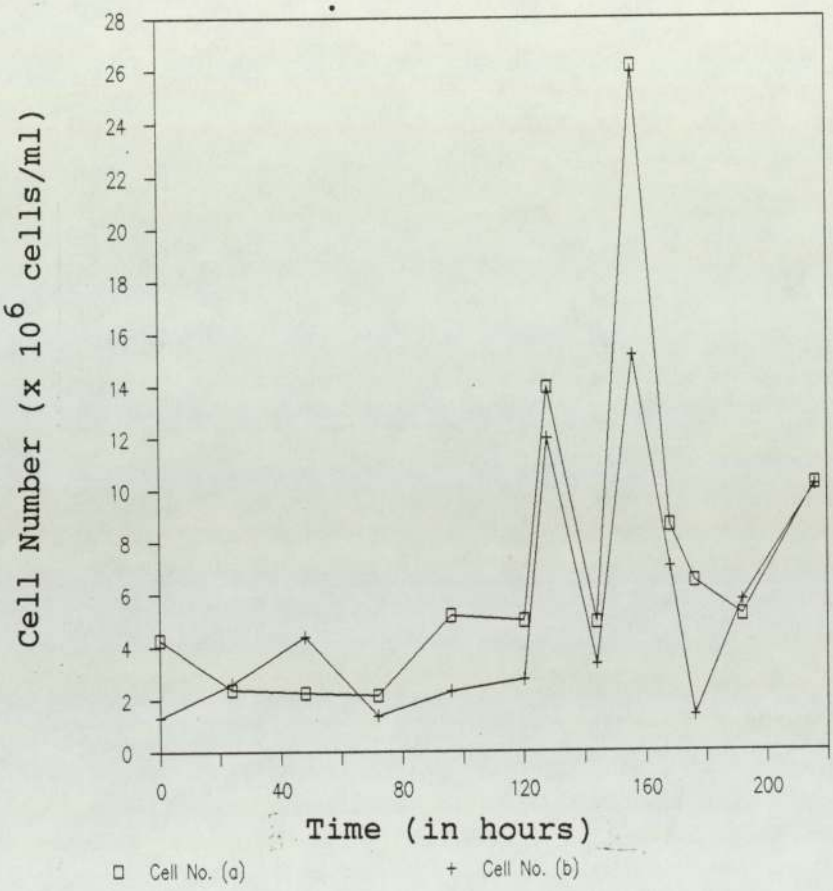
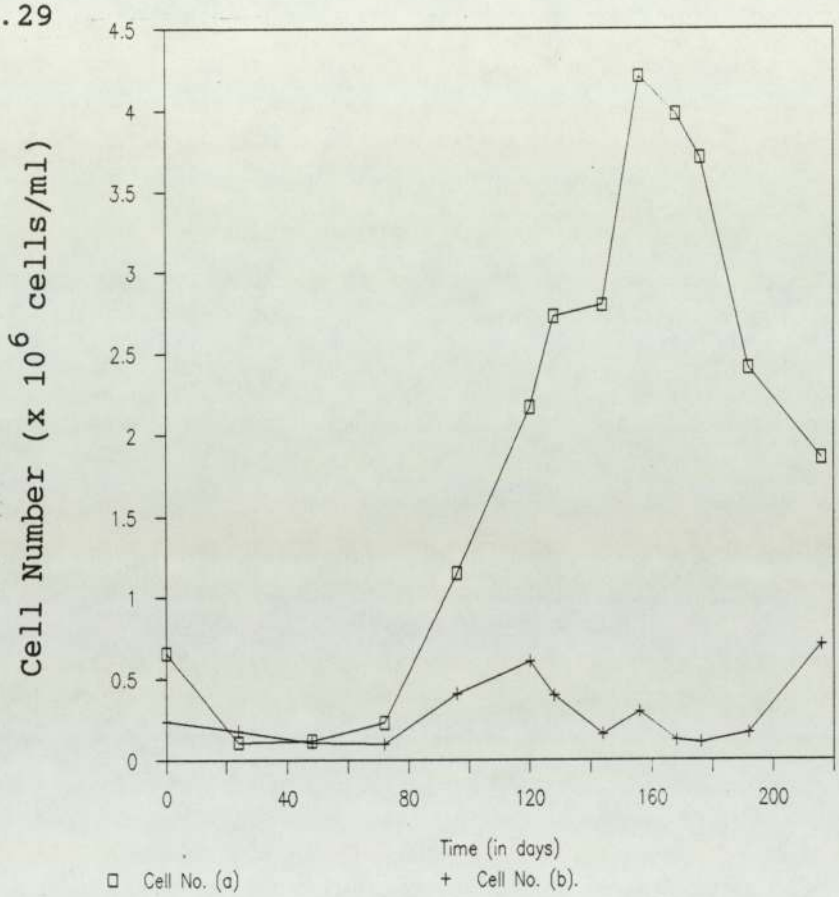


FIG.4.29.The Growth Characteristics of HL-60 cells, at a low cell density of $5x10^5$ cells/ml, in diffusion chambers implanted into CBA/Ca mice assessed over various time periods.

All figures are the mean value of 2 chambers which were constructed using 0.45μm filters and were implanted using a lateral incision site. The cell viability was >85% and the percentage of spontaneous differentiation was <10%. Standard error was <10%.

Figures show the results of two experiments a and b.

Fig 4.29



density was 5 x 10^6c/ml., the growth of the cells was very erratic with poor agreement between replicate chambers. There was still an initial growth lag before the cells commenced growth in the chambers. A plateau phase of growth was reached after 130 hours *in vivo*. The cell viability was in excess of 85% and the percentage of spontaneous differentiation was <13% for all the chambers. Similarly, when the cells were seeded at a lower density of 5 x 10^5cells/ml., the cell growth within the chamber was again erratic with poor agreement between replicate chambers. There was also a chamber in which the cells had not grown at all, approximately 1 out of 5 chambers did not support cell growth. This may have been because there were an insufficient population of cells in the diffusion chamber that were capable of surviving in the *in vivo* environment to sustain growth. Alternatively, Perkins *et al* (1984) have shown that HL-60 cells growing *in vitro* produce an auto-stimulatory factor which is necessary to sustain HL-60 cell growth, they will not grow at a very low density because there is insufficient of this factor. It was possible that there were too few remaining cells within the chamber to produce sufficient auto-stimulatory factor to stimulate further cell proliferation.

When HL-60 cells were used, at an initial cell density of 1 x 10^6cells/ml., the cell growth was reproducible (fig 4.23) with 4 days of log phase of growth and with good agreement between replicate chambers. This optimum cell

number was used in all further experiments.

4.4b. Results of pretreating the mice with cyclophosphamide prior to chamber implantation.

As outlined in section 1.6.5., it was shown that for the culture of many types of cells and tissues in diffusion chambers *in vivo*, it was necassary either to pretreat the host animals with cyclophosphamide or to irradiate them before implantation of the chamber. In addition, Lotem & Sachs (1978) demonstrated that the pretreatment of mice with cyclophosphamide prior to the implantation of the chamber, caused an inhibition of spontaneous *in vivo* differentiation of mouse myeloid cells in response to the macrophage and granulocyte inducing proteins. Therefore, experiments were performed to determine the effects of pretreatment of the mice with cyclophosphamide on the growth and differentiation of HL-60 cells cultured in diffusion chambers (Fig.4.30).

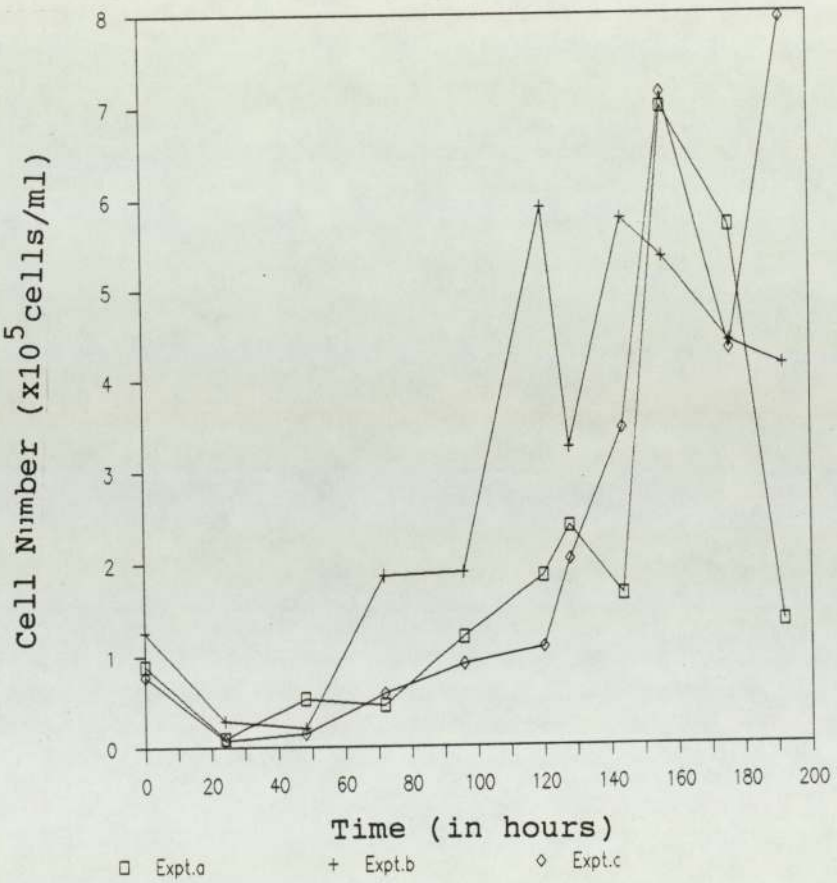
There was again an initial lag phase but thereafter the cells did not resume logarithmic phase of growth and the cell yield from each chamber was poor. There was also, not surprsingly, an increased number of infections within the individual chambers. The viability of the cells was less than had been previously observed in the chambers but was in excess of 70%.

FIG.4.30. Growth Characteristics of $1x10^6$ HL-60 cells/ml in diffusion chambers, implanted into CBA/Ca mice that had been pretreated with 200mg/kg Cyclophosphamide 24 hours prior to chamber implantation.

All figures are the mean value of 2 chambers which were constructed using 0.45µm filters and were implanted into miceusing a lateral incision site. The cell viability was >70% and the percentage of spontaneous differentiation was <10%.
Standard error was <10%.

Figures show the results of 3 experiments a, b and c

Fig 4.30



These results were in contrast to those of various other workers who routinely implanted diffusion chambers into cyclophoshamide-pretreated mice. Brevik & Benestad (1972) reported that mouse granulopoiesis was markedly enhanced when the diffusion chambers were implanted in irradiated- or cyclophosphamide - pretreated host mice. Laissue et al (1974) studied the effect of host irradiation on the growth of autologous bone marrow in diffusion chambers implanted into goats. They found that haemopoiesis was observed in both irradiated and non-irradiated hosts but the rate of proliferation was more rapid in the irradiated hosts and the cell counts were higher. It was suggested that the enhanced growth in the diffusion chambers might have been related to suppression of the hosts' immune response or to specific or non-specific modifications of the in vivo "culture medium" (hosts' body fluids diffusing into the diffusion chamber), for example, increased CSF levels.

Preisler et al (1978) and Gordon et al (1975) have suggested that irradiation of the host animal was necessary to support the growth and differentiation of human haemopoietic cells and that growth in non-irradiated hosts was poor and not reproducible. In contrast, Lotem & Sachs (1983) have reported no difficulty in culturing mouse MGI myeloid leukaemia cells in diffusion chambers in non-irradiated hosts, suggesting that allogeneic or

syngeneic cells can proliferate within a diffusion chamber in a non-irradiated host mouse whereas xenogeneic cells require host irradiation for good reproducible growth.

Schwartz et al (1983) and Chikkappa et al (1980) have cultured HL-60 cells and human granulocytes respectively in diffusion chambers in vivo. In both cases the diffusion chambers were implanted into an irradiated host mouse for 7 days and then, due to the imminent death of the host, the chambers were transferred to a newly irradiated host mouse to continue the culture period. This procedure would have increased the risk of infecting the implanted cells; but also has the advantage that the chamber filter membrane could be wiped free of any adherent mouse cells and tissues before being implanted into the second recipient. However, the results presented here demonstrate that it was possible to reproducibly culture HL-60 cells within a diffusion chamber in vivo without the pretreatment of the host animals with cyclophosphamide.

Therefore, the optimum growth conditions for HL-60 promyelocytic leukaemic cells within a diffusion chamber in vivo required a lateral incision site, serum free HL-60 cells at an initial cell density of 1×10^6c/ml. The optimum diffusion chamber was constructed by use of a 0.45μm filter membrane and was implanted into untreated CBA/Ca mice.

4.5. Comparison with L1210 leukaemia cell growth in diffusion chambers in vivo

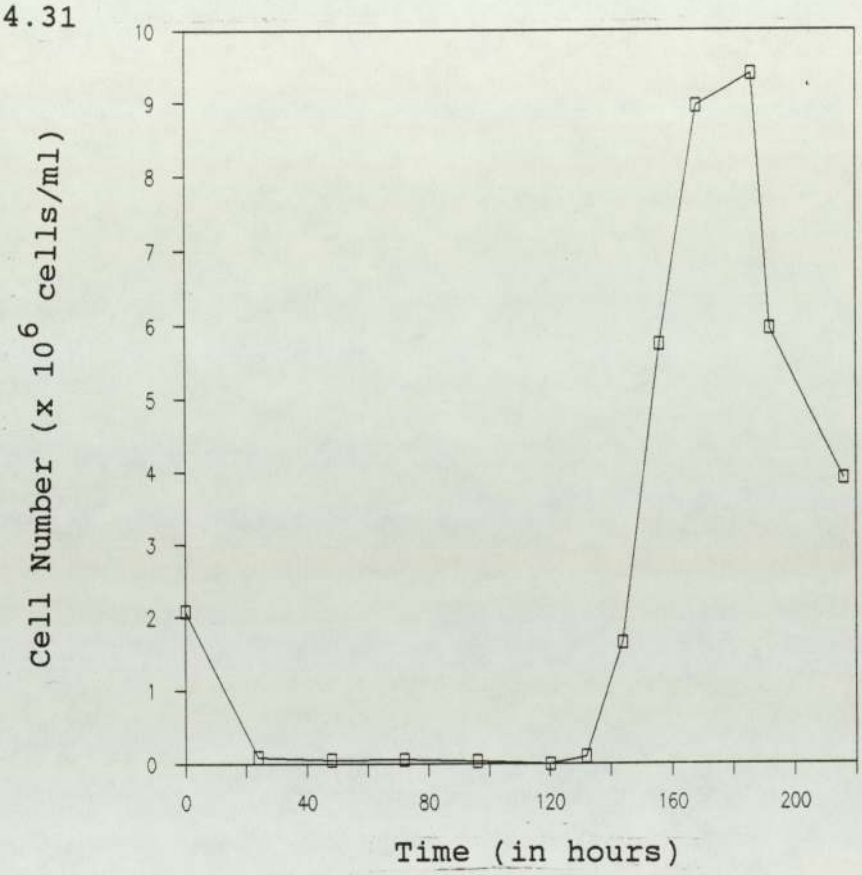
Anderson et al (1983) reported that L1210 cells could be propagated within a diffusion chamber in vivo (see section 1.6.7). Experiments were then performed to determine if the growth characteristics of the L1210 cells resembled those of HL-60 cells in diffusion chambers in vivo (as described in section 4.4), or whether they were similar to those reported by Anderson et al (1983), with immediate and rapid growth upon implantation of the chamber.

L1210 cells that were routinely passaged in 10% horse serum/RPMI in vitro were cultured initially within a diffusion chamber which was implanted into CBA/Ca mice. At an initial cell density of $5x10^5$ cells/ml, the growth of the cells in the chamber was very erratic and less than 50% of the cells were viable after 6 days (data not shown). An increase in the initial cell density to $2x10^6$ cells/ml resulted in some cell growth in the chamber, but again this was erratic and not reproducible (Fig 4.31). It was possible that this in vitro cell line might have grown in vivo if the chambers had been implanted into cyclophosphamide-pretreated mice, but this work was not continued. These results suggest that not all in vitro cell lines can be transferred to chambers for growth in vivo.

FIG.4.31.The Growth Characteristics of L1210 cells, at an initial cell density of $2x10^6$ cells/ml, in diffusion chambers implanted into CBA/Ca mice and assessed over various time intervals.

The diffusion chambers were constructed using 0.45μm filters and were implanted using a lateral incision site. All figures are the mean value of 2 chambers. The cell viability was >65% and the standard error was <10%.

Fig 4.31



The result of experiments using L1210 cells, that were harvested from ascites 4 days following ip injection of $2x10^6$ L1210 cells into female BDF1 mice, is shown in Figs 4.32-4.34. The growth characteristics of these cells in diffusion chambers and implanted into CBA/Ca mice were very similar to those observed for HL-60 cells (as described in section 4.4). When the cells were seeded at a low cell density of 1 x 10^5c/ml. (fig 4.32), and injected into chambers, which were constructed from 0.45μm filters, there was an initial cell loss during the first 48 hours <u>in vivo</u>. The cells then commenced growth and reached a plateau at 144-156 hours. The viability was in excess of 80% for the duration of the experiment. Seeding the cells at a higher density of 2 x 10^6c/ml. (fig 4.33) again resulted in a growth lag before the cells resumed growing. The cells reached a maximum cell density at 144 hours and thereafter the cell number did not decline significantly and remained at the maximum for the remainder of the experiment. A decrease of the innoculum cell density to 5 x 10^5c/ml. (fig 4.34) resulted in more reproducible growth, with similar characteristics to HL-60 cells at their optimum cell density (fig 4.23). The viability of the cells was in excess of 85% and, after an initial growth lag, the cells grew in logarithmic phase of growth and reached a plateau at 156 hours and decreased in number thereafter. Alteration of the filter pore sizes is shown in Figs 4.34 -4.36. As was observed with HL-60

FIG.4.32.The Growth Characteristics of L1210 cells, at an initial density of $1x10^5$ cells/ml, in diffusion chambers, implanted into CBA/Ca mice and assessed over various time periods.

L1210 cells were obtained from the peritoneal cavity of a BDF1 female mouse and were diluted to the appropriate density using saline. All figures are the mean value of 2 chambers, which were implanted using a lateral incision site and constructed using 0.45µm filters. The cell viability was >85% and the standard error was <5%.

Fig 4.32

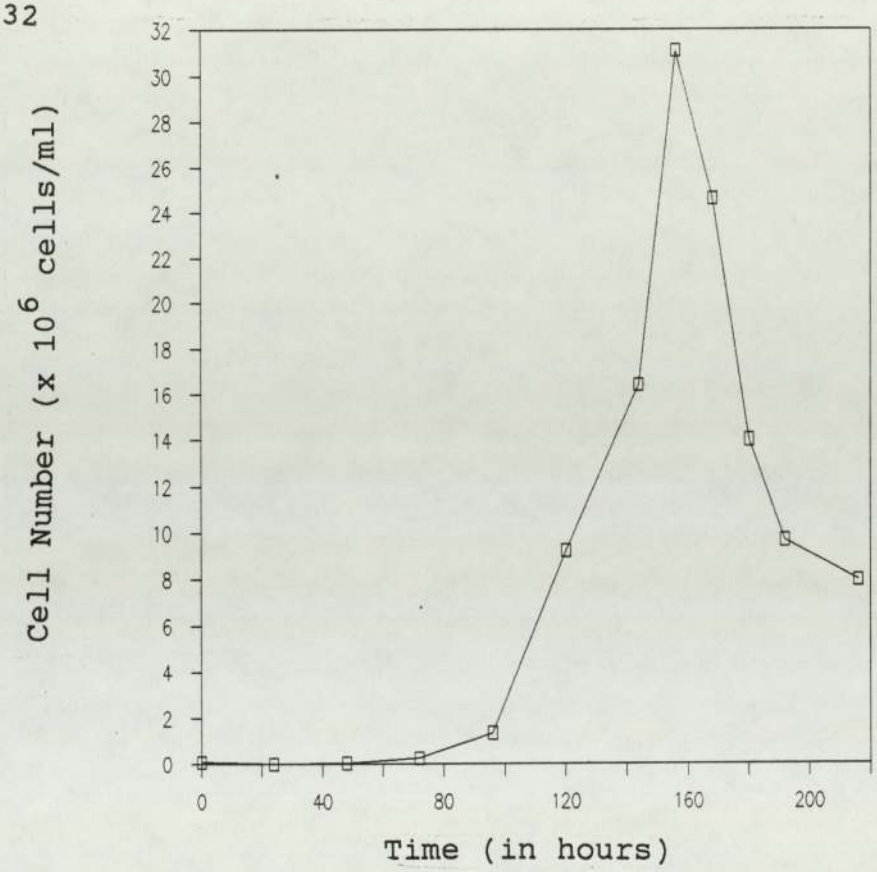


FIG.4.33. The Growth Characteristics of L1210 cells, at an initial density of $2x10^6$ cells/ml, in diffusion chambers implanted into CBA/Ca mice and assessed over various time periods.

L1210 cells were obtained from the peritoneal cavity of a BDF1 female mouse and were diluted to the appropriate density using saline. All figures are the mean value of 2 chambers, which were implanted using a lateral incision site and were constructed using 0.45μm filters. The cell viability was >85% and the standard error was <5%.

Fig 4.33

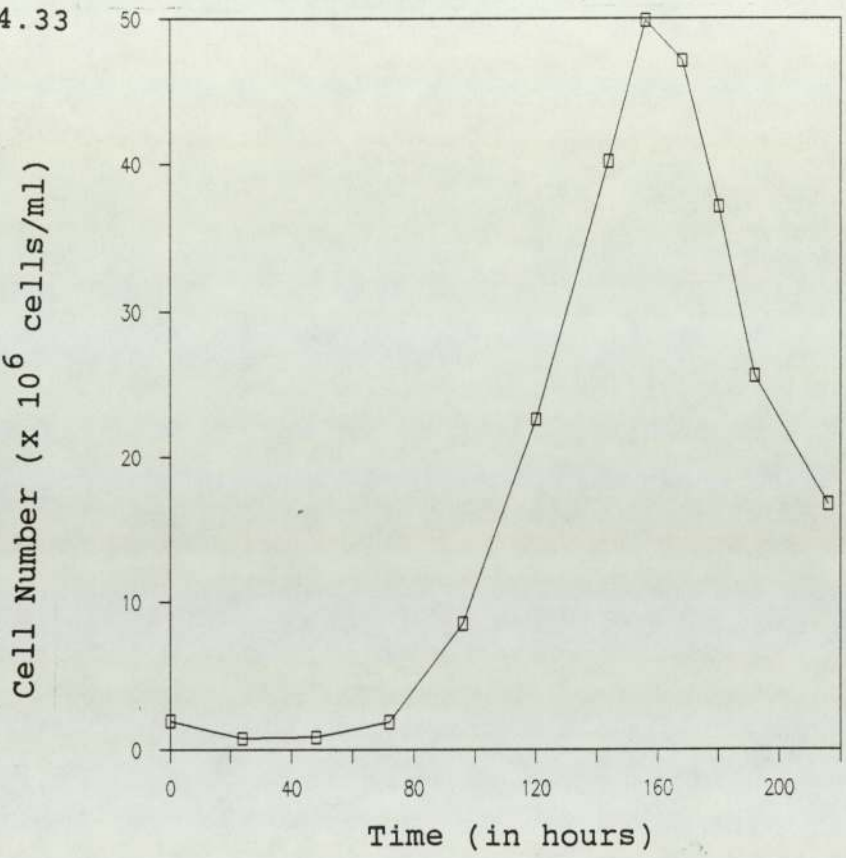


FIG.4.34. The Growth Characteristics of L1210 cells, at an initial density of $5x10^5$ cells/ml, in diffusion chambers implanted into CBA/Ca mice and assessed over various time periods.

L1210 cells were obtained from the peritoneal cavity of a BDF1 female mouse and were diluted to the appropriate volume using saline. All figures are the mean value of 2 chambers which were implanted using a lateral incision site and were constructed using 0.45µm filters. The cell viability was >85% and the standard error was <5%.

Fig 4.34

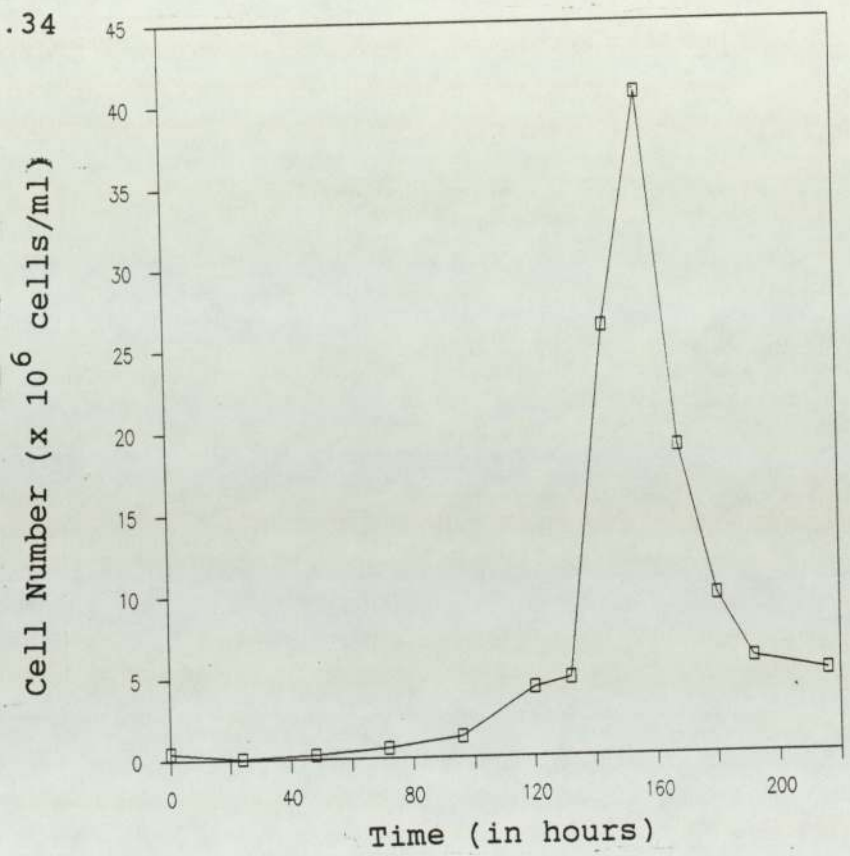


FIG.4.35.Growth of L1210 cells when cultured in a diffusion chamber which was constucted using 0.22µm filters and implanted into CBA/Ca mice and assessed over various time periods.

L1210 cells were obtained from the peritoneal cavity of a BDF1 female mouse and were diluted to the appropriate density using saline. All figures are the mean value of 2 chambers which were implanted using a lateral incision site. The cell viability was >85% and the standard error was <5%.

Figures show the results of two experiments a and b.

Fig 4.35

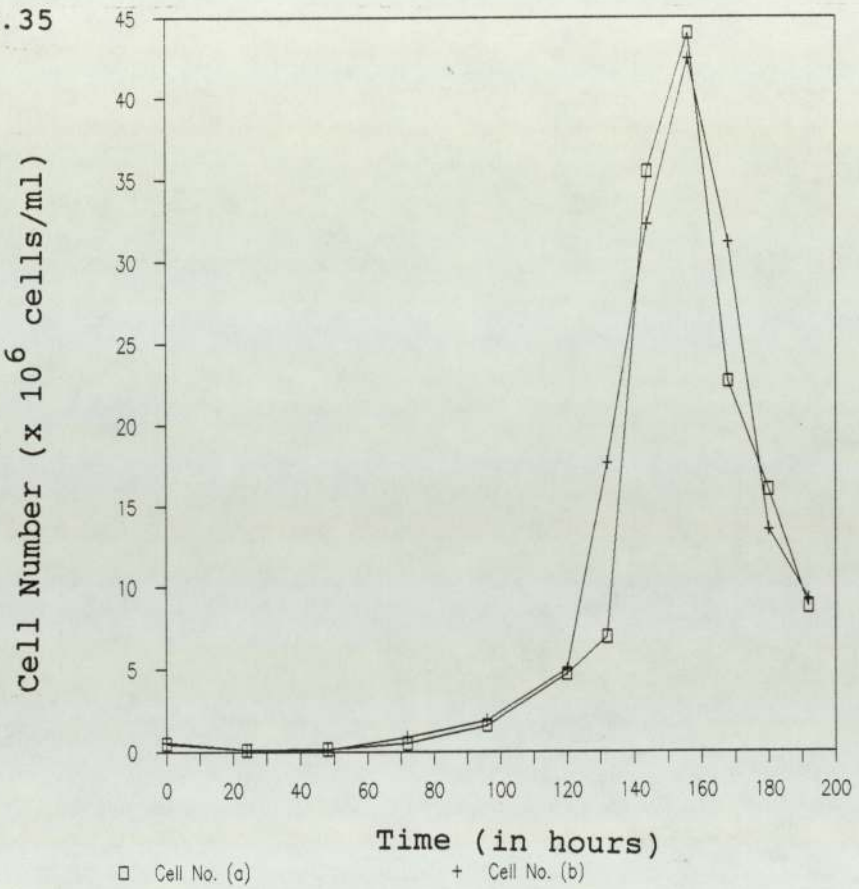
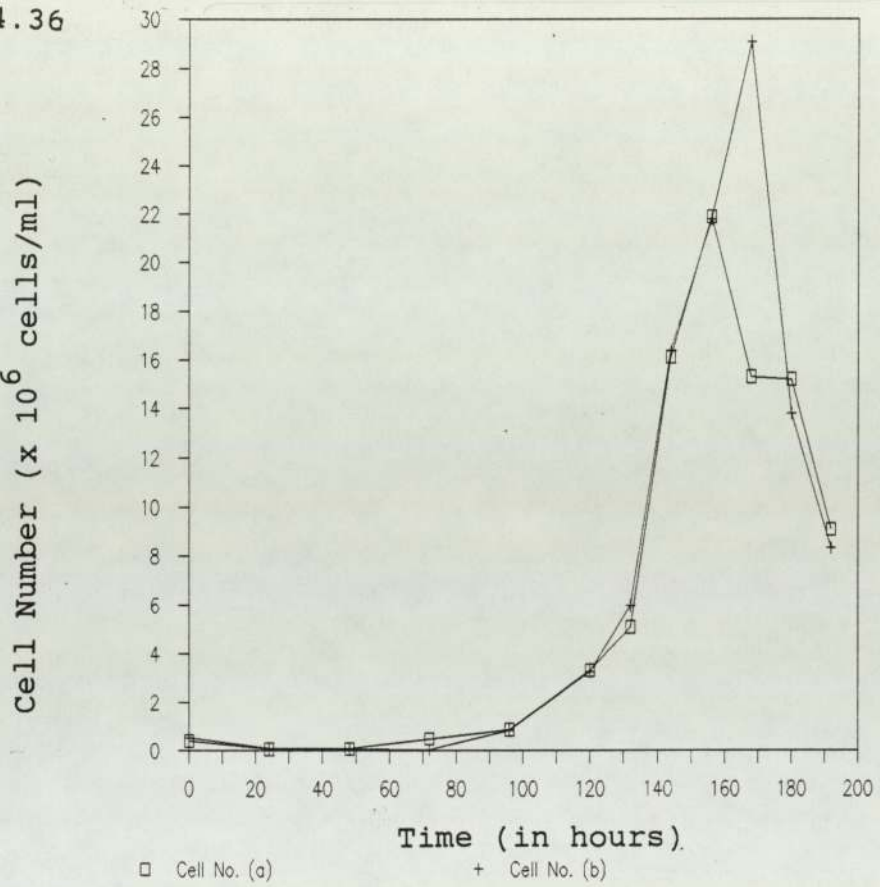


FIG.4.36.Growth of L1210 cells when implanted in a diffusion chamber which was constructed using 0.1um filters and implanted into CBA/Ca mice and assessed over various time periods.

L1210 cells were obtained from the peritoneal cavity of a BDF1 female mouse and were diluted to the appropriate density using saline. All figures are the mean value of replicate chambers which were implanted using a lateral incision site. The cell viability was >85% and the standard error was <5%.

Figures show the results of two experiments a and b.-

Fig 4.36



cells, the 0.45 and 0.22μm filters supported good reproducible cell growth, whereas the 0.1μm filters supported only poor growth with the cells never resuming log phase growth within the chambers.

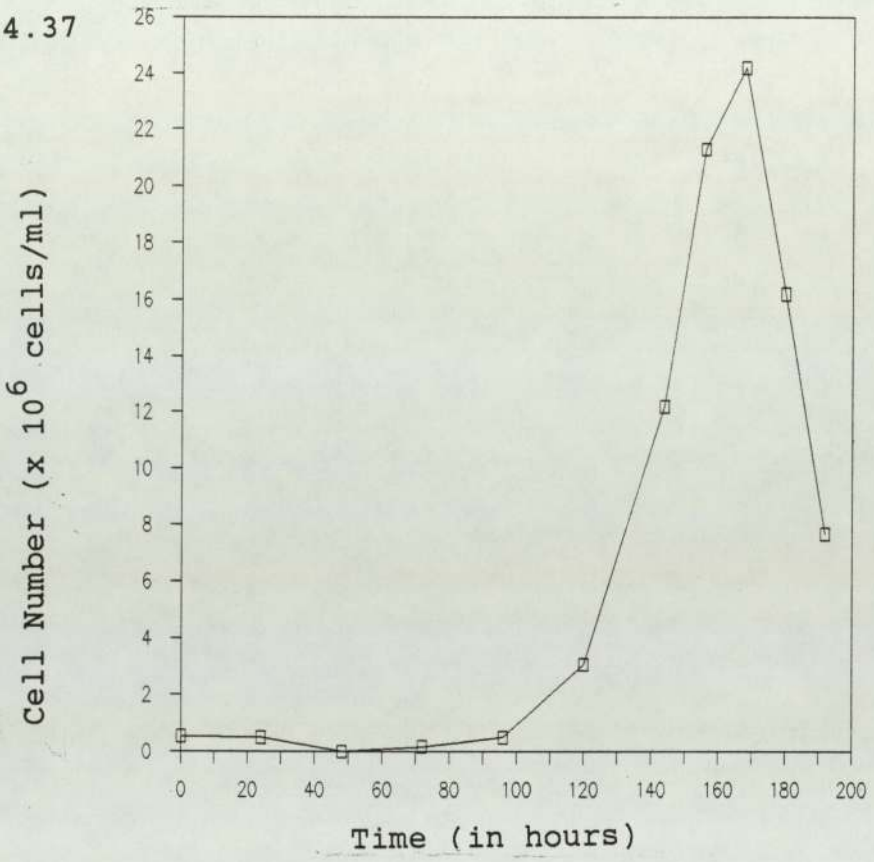
The initial growth lag observed always occurred when a lateral incision site was used. Anderson et al (1983) studied the effects of various doses of cyclophosphamide on the growth of in vivo L1210 cells in diffusion chambers implanted into BD2F1 mice. They used a central incision site and reported that the cells grew rapidly and immediately upon implantation and reached a plateau after 3-5 days in culture. To enable an accurate assessment of the effects of a single dose of cyclophosphamide on L1210 cell growth, only 100μl of cell suspension was injected into each chamber which allowed for rapid cell growth and ensured that the cells would not reach a plateau too early. Using this technique it was concluded that this provided reproducible information on drug effects and could be a valuable tool for designing clinically useful dose schedules.

Pretreatment of the mice with 200mg/kg cyclophosphamide 24 hours prior to implantation of the chambers did not alter the growth characteristics of the cells within the diffusion chambers (fig 4.37). As with HL-60 cells, implanted into cyclophosphamide-pretreated mice, there were an increased number of infections within the chambers

FIG.4.37.The growth of L1210 cells, at an initial density of $5x10^5$ cells/ml, cultured in a diffusion chamber, in mice that had been pretreated with 200mg/kg Cyclophosphamide 24 hours prior to chamber implantation.

All figures are the mean value of 2 chambers, which had been constructed using 0.45μm filters, and which were implanted using a lateral incision site. Standard error was <10%.

Fig 4.37



and no enhanced cell growth.

4.6. A pharmacokinetic study of the amounts of drugs attained in a diffusion chamber following the administration of a single ip injection of NMF, NEF, TMU,DBF, TMBF and HMBA

As described in section 4.4., the potential differentiating agents were to be administered to the animals after the chamber had been cultured *in vivo* for 5 days, when the implanted HL-60 cells were growing logarithmically. All the drugs were to be administered as a single ip injection at a dose that was 80% of the LD_{10}. This dose was chosen so that accurate comparisons could be made between the efficacy of the various drugs, at doses which were equitoxic and would not result in the death of any of the animals. However, after 4/5 days *in vivo*, the chambers became coated with mouse cells and tissues (Fig 2.4), and it was thought that this might have impeded the diffusion of the drugs across the filter menbranes of the chambers. Therefore, the blood and the chamber contents, of chambers that had been implanted for 1 and 5 days, were assayed after a single ip injection of drug to determine the amount of drug in the chambers over various time periods.

Fig. 2.4. Photograph to show a filter membrane of a diffusion chamber after it had been in vivo for 5 days and its surface was coated with adherent mouse cells.

Previous investigators who have examined the effect of various drugs on the growth of cells within the chambers have rarely studied the pharmacokinetics of the drugs they have tested and, therefore, do not know whether the drugs enter the chamber, and if so, for how long and at what concentration. The response of the implanted cells to the drugs may be due to the effects of the hosts' response to the drugs, rather than to the direct action of the drugs on the cells if the drug is unable to enter the chamber.

Anderson et al (1983), who studied the effects of cyclophosphamide on the growth of L1210 cells in diffusion chsmbers in vivo, did not determine whether the drug actually entered the chamber. Schwartz et al (1983), who examined the differentiating ability of anthracycline antibiotics on the growth of HL-60 cells in chambers, also did not determine whether these agents entered the chambers. In addition, Schwartz et al (1983) had cultured the HL-60 cells in agar in diffusion chambers and Gordon & Blackett (1976) had previously concluded that the presence of agar in a chamber substantially altered the response of the implanted cells to drugs (as outlined in section 1.6.7).

However, Selby & Steele (1982) studied the effect of Melphelan, Adriamycin and MeCCNU on the growth of human melanoma xenografts in agar in diffusion chambers. They

did examine the amount of Melphelan that entered the diffusion chamber and the blood using radio-labelled Melphelan; they found that the drug levels attained in the chamber were significantly lower than in the blood. They concluded that the Melphelan might have been held selectively in the cells rather than in the agar matrix which could have explained the low average Melphelan concentration for the chamber contents.

Experiments were performed to determine whether the potential differentiating agents could enter the diffusion chambers *in vivo*. The drug levels after a single ip dose of NMF, NEF, TMU and HMBA in both the blood and chambers are shown in figs 4.38-4.41.

Determination of the plasma concentration of NMF after administration of a single ip dose of 400mg/kg gave an area under the plasma concentration time curve of 4573ug h L^{-1} .The AUC value for the drug levels within the 1 day and 5 day-implanted diffusion chambers, which contained cells, were very similar (Table 4.3). These NMF blood concentrations are in agreement with those reported by Gescher *et al* (1982) who showed that the plasma elimination half-life of NMF in CBA mice was approximately 6 hours.

Likewise, the NEF levels in the blood and the chambers were similar with no significant difference between the

FIG.4.38.A Study of the Pharmacokinetics of a single intraperitoneal injection of 400mg/kg NMF.

Fig 4.38a - Blood levels of NMF determined in CBA/Ca mice that had been implanted with a chamber for 1 or 5 days (mg/L).
Fig 4.38b - NMF levels in chambers that had been implanted for 1 day (mg/L).
Fig 4.38c - NMF levels in chambers that had been implanted for 5 days (mg/L).

The chambers contained either HL-60 cells or RPMI 1640 medium alone; they were constructed using 0.45µm filters and implanted using a lateral incision site.

Fig 4.38a

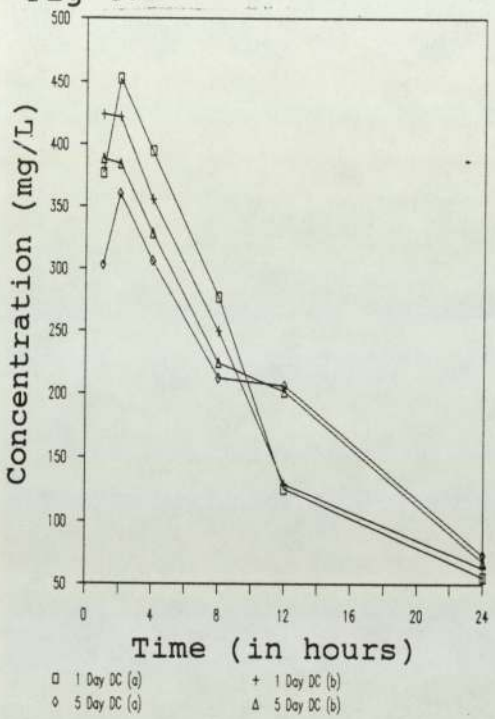


Fig 4.38b

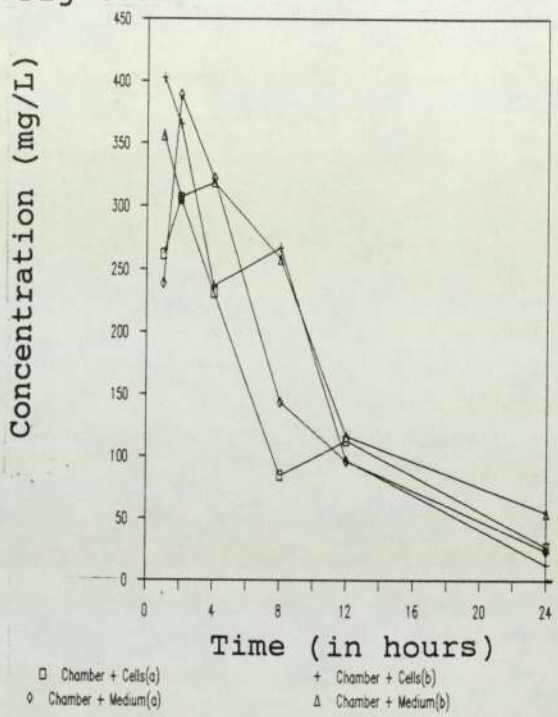
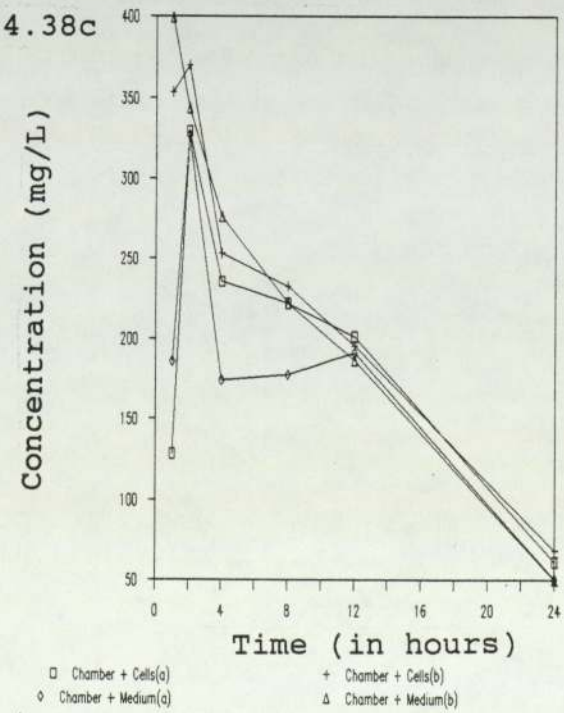


Fig 4.38c



Figures show the results of two experiments a and b.

FIG.4.39.A Study of the Pharmacokinetics of a single intraperitoneal injection of 350mg/kg NEF.

Fig 4.39a - Blood levels of NEF determined in CBA/Ca mice that had been implanted with a chamber for 1 or 5 days (mg/L).
Fig 4.39b - NEF levels in chambers that had been implanted for 1 day (mg/L).
Fig 4.39c- NEF levels in chambers that had been implanted for 5 days (mg/L).

The chambers contained HL-60 cells or RPMI 1640 medium alone; they were constructed using 0.45µm filters and were implanted using a lateral incision site.

Fig 4.39a

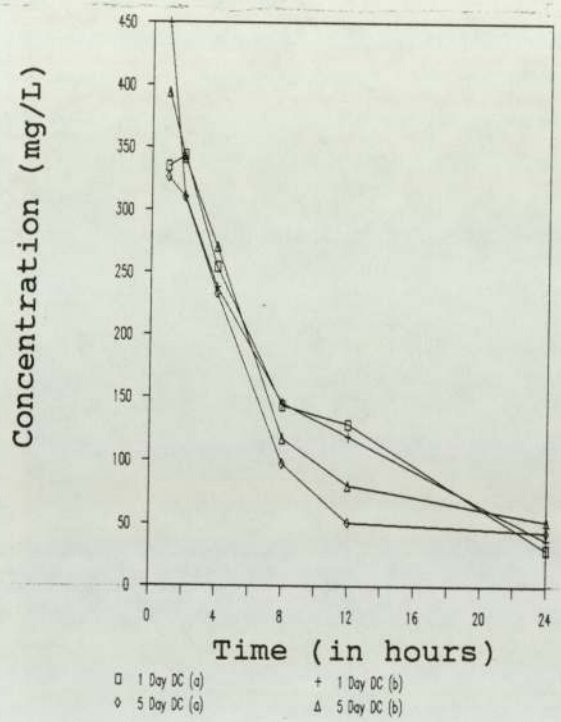


Fig 4.39b

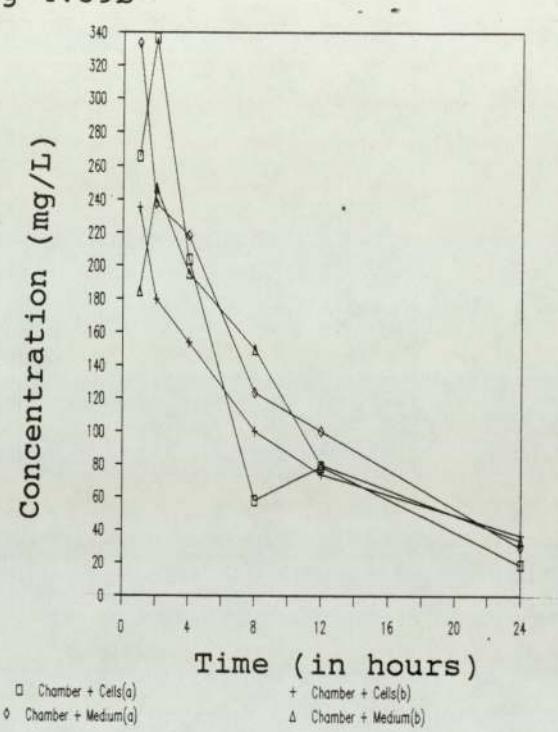
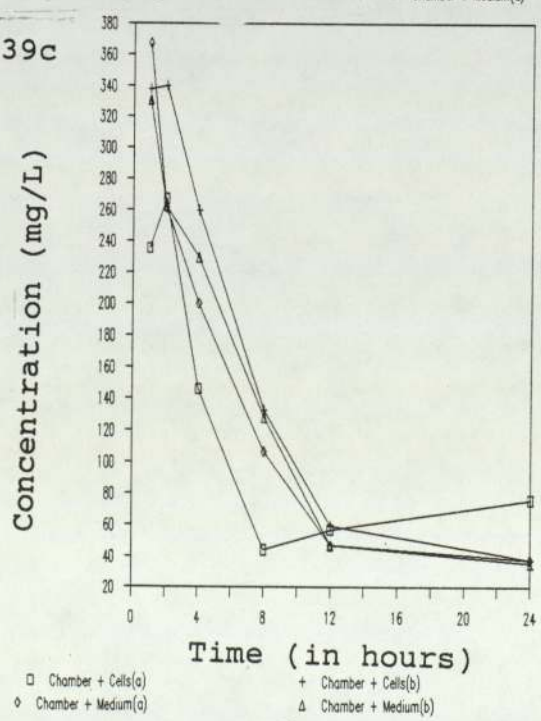


Fig 4.39c



Figures show the results of two experiments a and b.

FIG.4.40A Study of the Pharmacokinetics of a single intraperitoneal injection of 1600mg/kg TMU.

Fig 4.40a - Blood levels of TMU determined in CBA/Ca mice that had been implanted with a chamber for 1 and 5 days (g/L).
Fig 4.40b - TMU levels in chambers that had been implanted for 1 day (g/L).
Fig 4.40c - TMU levels in chambers that had been implanted for 5 days (g/L).

The chambers contained HL-60 cells or RPMI 1640 medium alone; they were constructed using 0.45µm filters and were implanted using a lateral incision site.

Fig 4.40a

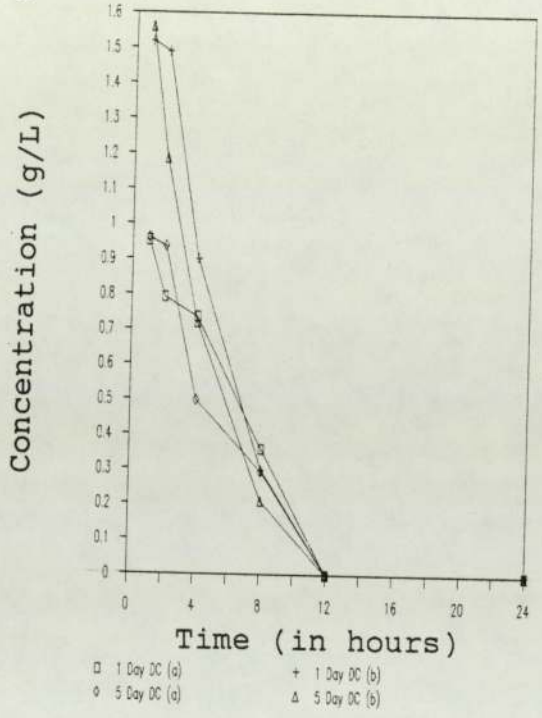


Fig 4.40b

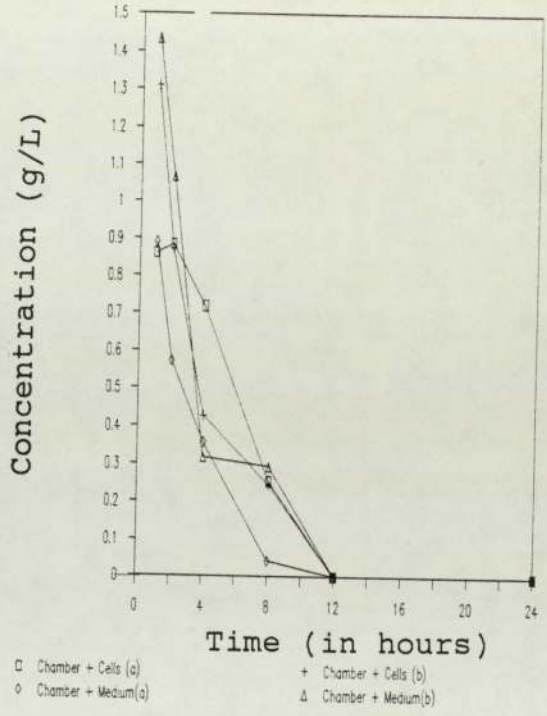
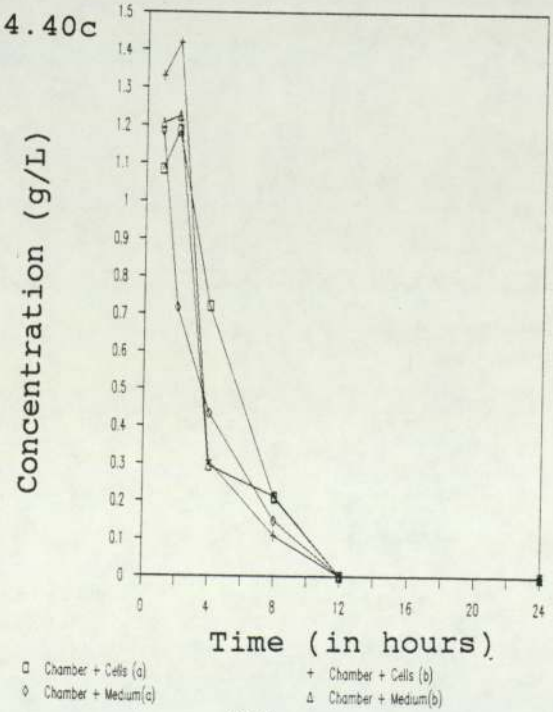


Fig 4.40c

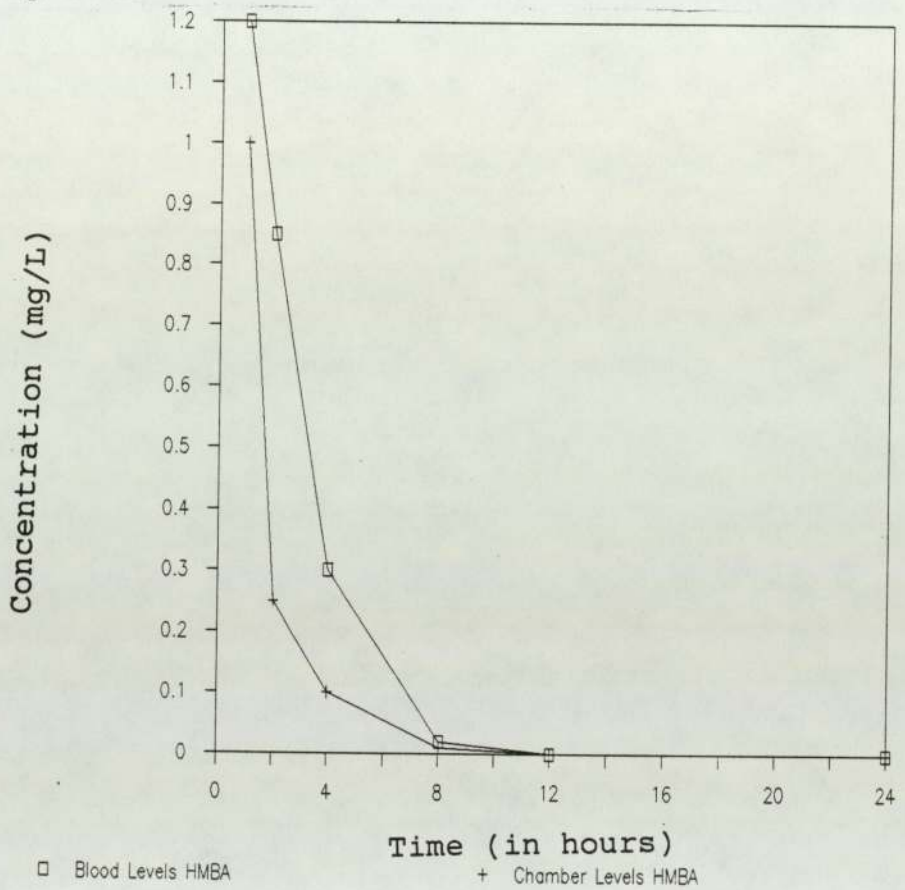


Figures show the results of two experiments a and b.

FIG. 4.41. A Study of the Pharmacokinetics of a single intraperitoneal injection of 2020 mg/kg HMBA.

Fig.4.41 - Blood and chamber levels of HMBA determined in CBA/Ca mice that had been implanted with a chamber for 5 days (mg/L).

Fig 4.41



levels obtained in the chambers that had been implanted for 1 and 5 days. The area under the plasma concentration time curve was 3404ug h L^{-1}. The AUC values for chambers that had been implanted for 1 and 5 days are shown in Table 4.3.

A single ip injection of 1600mg/kg TMU produced an area under the plasma concentration time curve of 6060ug h L. After 12 hours no TMU was detectable in the plasma by our GLC assay, however, after 6 hours there were increasing amounts of a metabolite which reached a maximum by 12 hours and then decreased and was undetectable by 24 hours. The area under the chamber concentration time curve for chambers which had been implanted for 1 and 5 days is shown in Table 4.3.

In contrast, no dibutylformamide (DBF) could be detected in either the blood or the chambers in time periods which ranged from 5 minutes to 24 hours after a single ip injection. Homogenates were then made of the liver, kidneys and peritoneal adipose tissue 2 hours after a single dose of DBF and any DBF in these tissues was extracted and assayed using GLC. Subsequently, the majority of the injected dose (approximately 95%) was found to be located in the adipose tissue with a small amount located in the liver. No DBF was detected in any of the diffusion chambers.

Table 4.3. AUC Values for NMF, NEF, TMU and HMBA

AUC Values (1 day chambers) $\mu g\ h\ L^{-1}$

Inducer	Blood Levels	Chamber + Cells	Chamber + Medium
NMF	4573	3272	3708
NEF	3404	2263	2697
TMU	6060	4620	4103

AUC Values (5 day chambers) $\mu g\ h\ L^{-1}$

Inducer	Blood Levels	Chamber + Cells	Chamber + Medium
NMF	4930	4386	4096
NEF	2791	2508	2450
TMU	5179	4889	4102
HMBA	3415	1675	NM

NM - not measured.
All figures are the mean values of 4 experiments. The mice were each administered a single ip injection of 80% of the LD 10 dose of each of the inducers. The blood and the chamber contents were assayed using GLC.

The drug levels of a single dose of 2020mg/kg HMBA were also measured in chambers that had been implanted for 5 days (Table 4.3). As was observed with TMU, HMBA was rapidly metabolised and after 12 hours no HMBA could be detected in the blood or in the chambers. The area under the plasma concentration time curve for the blood levels of HMBA was 3415ug h L and the value for the chamber was 1675 ug h L^{-1}(Table 4.3).

Therefore, it appeared that drug access to the chambers was not significantly impeded by the accumulation of mouse cells and tissues on the surface of the filter membranes, NMF, NEF, TMU and HMBA still gained access to the chambers. The presence of HL-60 cells within the chambers did not significantly alter the concentrations of drugs attained. This was in contrast to the work reported by Selby & Steele (1982) who showed that a chamber that contained cells had significantly decreased amounts of Melphelan as compared with the amounts in the blood. They did not determine the amounts in a cell-free chamber.

The results presented here were in concordance with those reported by Nettesheim et al (1966). They reported that cultures within a diffusion chamber could grow and survive in vivo for 2-4 weeks before there was an excessive growth of peritoneal cells on the outer surface of the filter membrane which then impeded the diffusion of nutrients and growth factors into or out of the chamber. Such cultures

could be removed from the host animal, wiped free of the adherent material and implanted into a second recipient where cell growth would continue.

The effect of a 4 day dosing regime on drug levels in the chambers was next determined (Table 4.4). Because the potential inducers of differentiation were to be administered for 4 consecutive days - whilst the cells were in log phase growth conditions, it was necessary to determine whether there was an accumulation of the inducers within the chambers. Administration of 400mg/kg NMF, 350mg/kg NEF, 1600mg/kg TMU and 2020mg/kg HMBA for 4 consecutive days did not result in the accumulation of any of the inducers either in the blood or in the chambers which had been implanted for 5 days and which contained serum-free HL-60 cells . Neither TMU or HMBA were detected in the blood or the chambers after 24, 48, 72 or 96 hours and there were only minimal amounts of NMF and NEF detected.

Table 4.4. A 4 Day Regime of Dosing to Determine Whether the Drugs Accumulate within the Chamber.

Table 4.4a. NMF Levels in the blood and in the chamber.

Time (hr)	Blood Levels NMF (mg/L)		Chamber Levels NMF (mg/L)	
24	89.2	73.4	35.4	39.3
48	88.1	94.6	30.1	30.0
72	88.0	74.2	82.5	61.6
96	122	120	43.0	47.3

Table 4.4b. NEF Levels in the blood and in the chamber.

Time (hr)	Blood Level NEF (mg/L)		Chamber Levels NEF (mg/L)	
24	50.4	41.4	28.5	26.2
48	98.9	74.3	85.0	60.1
72	132	96.4	110	82.4
96	73.4	80.2	52.8	60.9

All figures are the mean values of 4 experiments. The mice were injected with 400mg/kg NMF and 350mg/kg NEF for 4 consecutive days and the blood and chamber contents were assayed for the inducers using GLC. All the mice contained a chamber that had been implanted for 5 days.
No TMU or HMBA were detected in the blood or in the chambers after 12 hours.

4.7. Attempts to model the in vivo drug exposures by the use of in vitro conditions of cell culture.

The work presented in section 4.6. showed the exposure times of the HL-60 cells, in diffusion chambers, to the various drugs. The amounts of drugs in the diffusion chambers decreased rapidly, and both TMU and HMBA were undetectable in the chambers by 12 hours, whereas there were minimal amounts of NMF and NEF remaining after 24 hours. The *in vitro* responses of the cells to the drugs (shown in section 4.1) was for a continuous exposure of the cells to the drugs; and therefore was not representative of the drug exposure times that occurred *in vivo*. Various experiments were thus performed in an attempt to model the *in vivo* drug exposures to HL-60 cells in *in vitro* culture to determine whether differentiation could be induced *in vitro*, with similar exposures to the drugs that occurred *in vivo*.

The *in vivo* regime of dosing involved 4 consecutive administrations of 80% of the LD_{10} dose of the various gaents (as outlined in section 4.6). Therefore the *in vitro* modelling was designed similarly, with the agents being administered for 4 consecutive days and the percentage of differentiation assessed 24 hours after the final "dosing".

The HL-60 cells were initially exposed to a drug concentration that was the optimum concentration for the induction of terminal differentiation of HL-60 cells *in vitro* (determined and discussed in section 4.1). The inducers were added for 12 hours/day and the percentage of differentiation was assessed on day 5. The inducers were added at concentrations of 170mM NMF, 80mM NEF, 8mM TMU and 2mM HMBA and were all able to induce significant terminal differentiation of HL-60 cells, although they were only present for half the cell cycle (table 4.5).

Although previous investigators had reported that it was necessary to expose HL-60 cells to inducers of differentiation for at least 24 hours before commitment to differentiation would occur (Fibach *et al*, 1982., Tarella *et al*, 1982), described in section 4.2. Tsiftsoglou *et al* (1985) reported that exposure of HL-60 cells, with a cell doubling time of 34-36 hours, to optimum *in vitro* differentiating concentrations of DMSO and retinoic acid for only 8 hours/day was sufficient to commit the cells to differentiate. Yen (1985) and Yen & Albright (1984) have extensively studied the exposure times of agents that were required to commit HL-60 cells to differentiate (outlined in section 1.5 and 4.2). They demonstrated that HL-60 cells could retain a memory of the exposure to the inducer, and on subsequent exposure to the inducer would then differentiate. This could provide an explanation for the differentiation observed after only 12 hours exposure

Table 4.5. Experiments using the optimum differentiating concentration of the inducers.

Inducer	No.c/ml (x100)	% Viability	% NBT	% Phagocytosis
Control	830	94.3	3.5	2.1
NMF	480	92.2	73.8	67.3
NEF	415	90.1	79.4	69.5
TMU	476	91.1	64.0	52.7
DBF	309	90.4	58.8	56.9
HMBA	456	93.2	67.8	58.9

Concentrations used were: 170mM NMF, 80mM NEF, 1mM DBF 8.5mM TMU and 2mM HMBA. All the inducers were added for 12 hours/day for 4 consecutive days and the percentage of differentiation was assessed on day 5. All figures are the mean values of 4 experiments and the standard error was <6%

to the various agents, if the HL-60 cells were able to retain a memory of the exposure to the agents and on subsequent administration they were then able to differentiate.

In further experiments, HL-60 cells, that were in the logarithmic phase of growth, were exposed to the maximum concentration of the agents that was measured *in vivo*, that is 7mm NMF, 5mM NEF, 12mM TMU and 2mM HMBA (Table 4.6). The inducers were added at these concentrations for 12 hours/day for 4 consecutive days and assays of differentiation were performed on day 5. Only those cells that had been exposed to TMU and HMBA showed any significant differentiation to granulocytes; NMF and NEF had no differentiating effect on the cells at these concentrations. These results were in accordance with the work presented by Yen & Albright (1984) who demonstrated that HL-60 cells could retain a memory of exposure to the inducers, providing that the inducers were present at an optimum *in vitro* differentiating concentration, and subsequent exposures to the inducers would result in commitment of the cells to differentiate (as described in section 4.2 and 4.7).

Therefore, in these experiments, it appeared that the first exposure of HL-60 cells to the drugs induced a precommitment memory as outlined by Yen & Albright (1984). Subsequent drug exposures then induced the differentiation

Table 4.6. Experiments using the in vivo concentrations of the inducers.

Inducer	No.c/ml. (x100)	% Viability	% NBT	% Phagocytosis
Control	783	98.7	6.3	1.4
NMF	771	96.9	7.2	2.0
NEF	794	97.4	6.3	0.8
TMU	404	81.8	58.7	34.3
HMBA	210	63.6	55.6	35.8

Concentrations used were : 7mM NMF, 5mM NEF, 12mM TMU and 5mM HMBA. The inducers were added for 12 hours/day for 4 consecutive days and the percentage of differentiation was assessed on day 5. All figures are the mean values of 4 experiments and the standard error was <5%.

of those cells and concomitantly a further fixed fraction of cells would obtain a precommitment memory of the exposure to the inducer, reminiscent of Von Melchner & Hoffken (1985)"....the probability of each cell to differentiate and at the same time cease dividing increases with each generation in the presence of a chemical inducer".

Although these experiments do imitate, to some extent, the in vivo drug exposures; they are not truely representative of the in vivo situation. In a diffusion chamber in vivo, the cells are exposed to decreasing amounts of the drugs over each time period. The total exposure time of the drugs to the cells was determined by calculation of the area under the plasma concentration time curve. These values were then used to expose the HL-60 cells, in vitro, to an equivalent amount of drug as was measured in vivo. The AUC values were determined for diffusion chambers that had been implanted for 5 days.

An equivalent drug exposure time was calculated for the exposure of HL-60 cells to NMF, NEF, TMU and HMBA for 6 hours/day which had an equivalent AUC value as had been determined in vivo ie for NMF, the AUC value was 4578.775 mg h L this gave an equivalent AUC value if the cells were exposed to 13mM NMF for 6 hours/day (table 4.7). Likewise, for the other inducers, exposing the cells to 5mm NEF, 7mM TMU and 2mM HMBA for 6 hours/day gave an

Table 4.7. Experiments using the Equivalent AUC Values.

Inducer	No.c/ml. (x100)	% Viability	% NBT	% Phagocytosis
Control	1550	98.5	4.9	2.2
NMF	1370	94.2	11.0	8.4
NEF	1400	96.4	11.4	7.4
TMU	860	97.2	37.5	33.6
HMBA	1240	89.7	12.1	11.0

Concentrations used were : 13mM NMF, 5mM NEF, 7mM TMU and 2mM HMBA. The inducers were added for 6 hours/day for 4 consecutive days and the percentage of differentiation was assessed on day 5. All figures are the mean values of 4 experiments and the standard error was <6%.

equivalent AUC value as was determined for the respective inducers diffusion chambers *in vivo*. The cells were exposed to the inducers for 4 consecutive days and the percentage of differentiation was assessed on day 5.

The cells that were exposed to TMU showed some *in vitro* granulocytic differentiation with only 6 hours exposure to the drug / day. There was approximately 37% granulocytic differentiation as was measured by assays of NBT and phagocytosis and the cell viability was >90%. The cells treated with NMF, NEF or HMBA did not show any significant increases in differentiation, the cell viability was also >90% and the cell numbers were comparable to the control cell numbers, whereas the cell numbers of the TMU-treated cells were significantly lower than the control cell numbers. It was interesting to observe that exposure of the cells to 2mM HMBA for 12 hours/day for 4 consecutive days was sufficient to induce differentation, whereas exposure of the cells to the same concentration for only 6 hours/day could not induce differentiation of the cells.

Further *in vitro* experiments were performed using HMBA and TMU. As described in section 1.2.3., HMBA has recently entered clinical trials as a potential differentiating agent. It was administered by continuous iv infusion for 120 hours and no objective tumour responses were noted. TMU was further assessed as it was possible to attain a concentration of 12mM in a chamber *in vivo*; and the

optimum concentration required for inducing differentiation *in vitro* was 8mM (as mentioned in section 4.1c). This agent was therefore considered a potential candidate for inducing the differentiation of HL-60 cells *in vivo*. The maximum amounts of NMF and NEF that were determined in the chambers *in vivo* were 7mM and 3.5mM respectively and since the optimum concentrations required for the induction of terminal differentiation *in vitro* were 170mm and 80mM respectively, it was unlikely that either NMF or NEF would be capable of inducing the terminal differentiation of HL-60 cells *in vivo*.

However, both TMU and HMBA were rapidly metabolised *in vivo* (as described in section 4.6), the cells within the diffusion chamber would only be exposed to a differentiating concentration of inducer for a short time period. Therefore, in an attempt to reproduce this *in vivo* situation, HL-60 cells were exposed *in vitro* to the maximum concentrations of TMU and HMBA that were measured *in vivo* for a time period that gave an AUC value equivalent to that calculated for the drug levels in diffusion chambers *in vivo* ie 12mM TMU for 3.5 hours/day; and 5mM HMBA for 1hour 40 minutes/day. This was also administered for 4 days. (Table 4.8). The cells exposed to TMU showed significant amounts of differentiation to granulocytes, with an average of 61.30% NBT positive cells and 48.67% of cells capable of phagocytosis. The average cell viability was >85% and the cell numbers were

Table 4.8. Experiments using the maximum in vivo concentrations and equivalent AUC values.

Inducer	No.c/ml. (x100)	% Viability	% NBT	% Phagocytosis
Control	1100	93.2	5.2	3.2
TMU	670	85.6	61.3	48.7
HMBA	980	81.4	13.5	10.8

Concentrations used were : 12mM TMU for 3.5 hours/day and 5mM HMBA for 1 hour 40 minutes/day. The inducers were added for 4 consecutive days and the percentage of differentiation was assessed on day 5. All figures are the mean values of 4 experiments and the standard error was <10%.

significantly lower than those of the untreated control cells. In contrast, the cells that were exposed to HMBA showed no significant differentiation. It is unclear why TMU should be capable of inducing differentiation using this regime and HMBA be incapable of such induction. The TMU was present at a more toxic concentration of 12mM for a slightly longer time period than the HMBA. 5mM HMBA did not induce marked cytotoxicity whereas 12mM TMU is more toxic.

Langdon & Hickman (1987) have suggested that the toxicity of a potential differentiating agent plays a vital role in determining whether a drug will be able to induce the terminal differentiation of HL-60 cells. They reported that the optimum concentration, of a range of differentiating agents, for the <u>in vitro</u> differentiation of HL-60 cells was at a concentration that was marginally below the concentration that was toxic to the cells. Therefore, as TMU was exposed to the HL-60 cells at a sub-toxic concentration of 12mM the drug was able to induce the terminal differentiation of the cells. HMBA, on the other hand, was exposed to the cells at a concentration of 5 mM, which was not as toxic to the cells (as mentioned in section 4.1e) and therefore was not able to induce the differentiation of the cells.

The clinical trial that involved HMBA (as described in section 1.2.3.) had attempted to maintain a concentration of HMBA in the plasma of 2mM for 120 hours. As mentioned previously (section 1.4., 1.5.,4.7.) various investigators have reported that HL-60 cells require 24 hours exposure to the inducer to commit them to differentiate; Langdon & Hickman (1987) have suggested that the inducers must also be present at a sub-toxic concentration before the induction of differentiation can occur. Therefore, although the trial that involved HMBA exposed the cells to the drug for a sufficient time period for commitment to occur, the drug might not have been present at a sub-toxic concentration which was also crucial if the drug was to act as a differentiating agent.

The results presented in this section suggest, that for differentiation therapy, an alternative regime of dosing to a continuous infusion might be repetitive regimes of dosing, with the cells exposed to sub-toxic amounts of the inducers. However, for cytotoxic chemotherapy, Anderson et al (1983) showed that a single dose of cyclophoshamide was more cytotoxic to L1210 cells, in diffusion chambers implanted into mice, than the administration of fractions of this dose.

4.8. In Vivo Differentiation.

Administration of a 4 day regime of dosing schedule of 80% of the LD_{10} dose of NMF, NEF, TMU, DBF, TMBF and HMBA to serum-free HL-60 cells in a diffusion chamber implanted into CBA mice is shown in tables 4.9 and 4.10.

The viability of the cells in the diffusion chambers was greater than 80%. The percentage of spontaneous granulocytic differentiation, as measured by NBT activity, ranged from 5-16% in the control diffusion chambers. There was no cellular NSE activity measured in any of the chambers. Some cells in the control chambers exhibited a high percentage of spontaneous differentiation which might have been due to inadequate removal of the foetal calf serum before the cells were injected into the chamber. Prior to the administration of the inducers, the mice were weighed daily to record any change in weight and appropriate adjustments made in the dosage.

In both the NMF and the NEF-treated mice, the percentage of granulocytic differentiation was comparable to that measured in the HL-60 cells of the control mice, and ranged from 5.33-18.1%. The cell viability was greater than 80% and the cell numbers were lower in the NMF and NEF-treated chambers than those determined in the diffusion chambers in the control mice, suggesting that

In Vivo Differentiation

Table 4.9aA 4 Day Regime of Dosing using 400mg/kg NMF, 350mg/kg NEF and 1600mg/kg TMU.

Inducer	No.c/ml. (x100)	% Viability	% NBT	% MoAb's
Control	6650	95.1	16.1	10.6
NMF	1190	89.8	15.5	11.4
NEF	2940	86.3	14.4	8.7
TMU	2010	84.5	50.4	48.3

Table 4.9bA 4 Day Regime of Dosing using 400mg/kg NMF, 350mg/kg NEF and 1600mg/kg TMU.

Inducer	No.c/ml. (x100)	% Viability	% NBT	% MoAb's
Control	13100	93.7	4.9	2.6
NMF	1570	89.8	10.4	5.8
NEF	5900	92.8	12.4	10.3
TMU	1390	90.3	47.3	41.3

CBA/Ca mice that contained a chamber that had been implanted for 5 days, were injected with the inducers for 4 consecutive days. The chambers were removed for assessment of HL-60 cell differentiation 24 hours after the final injection. All figures are the mean values of 4 mice.
MoAb's - monoclonal antibodies.

Table 4.10. A 4 Day Regime of Dosing using 150 mg/kg DBF, 10mg/kg TMBF, 12.5 mg/kg TMBF and 2020mg/kg HMBA.

Inducer	No.c/ml. (x100)	% Viability	% NBT	% MoAb's
Control	12240	93.2	5.8	4.3
DBF	1460	90	4.5	3.0
TMBF (10)	10670	83.2	9.7	6.7
TMBF (12.5)	10400	87.7	8.6	7.5
HMBA	3303	92.3	37.2	35.3

CBA/Ca mice that contained a chamber that had been implanted for 5 days, were injected with the inducers for 4 consecutive days. The percentage of differentiation was assessed 24 hours after the final injection. All figures are the mean values of 4 mice.
Non-specific esterase was negative for all samples.

both inducers have had some toxic but not differentiating effects on the leukaemic cells.

In contrast, the administration of 4 daily doses of TMU induced approximately 50% of the cells to differentiate to granulocytes. Similarly, HMBA also induced the *in vivo* terminal differentiation of the implanted HL-60 cells. However, the amount of differentiation was lower than that measured for TMU with only 37% of cells induced to differentiate.

TMBF was tested *in vivo* at two concentrations - 12.5 and 10 mg/kg. 12.5mg/kg was the LD_{10} and allowing for adverse drug effects, which often occurred with this compound, a lower dose of 10mg/kg was also tested. It was interesting to note the different LD_{10} doses of DBF and TMBF in the mice. These two compounds are isomers and, therefore, have the same molecular weight, which has been suggested to be important in the induction of differentiation *in vitro* (Langdon & Hickman, 1987). However, TMBF was approx ten times more toxic *in vivo* than DBF - with an LD_{10} of 12.5mg/kg in CBA mice whereas the LD_{10} for DBF was approx 170mg/kg. Both these compounds had very similar dose-response curves *in vitro* and the optimum *in vitro* differentiating concentration was 1mM.

These observations that both TMU and, to a lesser extent, HMBA, could induce the terminal differentiation of HL-60

cells *in vivo* were in concordance with the *in vitro* results presented in section 4.7.

It was possible to attain *in vivo* concentrations of TMU and HMBA that were sub-toxic to the implanted cells and that were similar to the concentration required for the differentiation of HL-60 cells *in vitro*. For the other drugs tested, the amounts that were measured *in vivo* were too far below the sub-toxic concentration that was required to induce differentiation *in vitro* and therefore these drugs could not act as differentiating agents *in vivo*.

These results also suggest that it is possible to commit the cells to differentiate without requiring the continual presence of the inducer, as suggested by Fibach *et al* (1982), but by dosing the animals on consecutive days with sub-toxic doses of the potential differentiating agents.

This raises an interesting question : whether the percentage of differentiation induced would be increased by the administration of either TMU or HMBA twice daily for 4 consecutive days? This would provide toxic peak levels twice/day and, as both TMU and HMBA were undetectable after 12 hours, may not be too toxic to the mice.

Only a few other investigators have examined the possibility of inducing differentiation *in vivo*. Some interesting findings were reported by Fauerholdt & Jacobsen (1975) who examined whether the maturational defect *in vivo* in acute leukaemia was due to environmental or cellular factors. They implanted diffusion chambers, which contained human acute myeloid leukaemia cells, into both irradiated and non-irradiated hosts to observe the growth and differentiation of these cells. Their data showed there was no significant differences between cell growth in irradiated and non-irradiated hosts and provided evidence supporting the concept of AML as a disturbance of cellular maturation which is due to cellular and not to environmental factors. Similarly, Steele *et al* (1977) cultured normal and acute myelocytic human leukaemia cells in diffusion chambers *in vivo* again to study any spontaneous differentiation. It was demonstrated that the human bone marrow cells showed an orderly pattern of growth and differentiation whereas the leukaemic cell population continued proliferation without differentiation, although normal appearing granuloctes were observed indicating that normal granulocytic precursor cells are present even in leukaemic marrow.

In contrast, Ganser *et al* (1985) utilised this tcchnique to determine whether cells from 3 patients could be induced to differentiate in a normal environment, without the addition of any differentiating agents. They

demonstrated that the cells from 2 out of the 3 patients did, in fact, differentiate along the granulocytic and megakaryocytic pathways and concluded that environmental factors do play a role in the maintenance of the leukaemic phenotype.

Lotem & Sachs (1979, 1984) have studied *in vivo* differentiation of HL-60 cells in diffusion chambers implanted into SJL mice. They reported that *in vivo* differentiation of serum-free HL-60 cells could be induced by several ip injections of Actinomycin D, but provided no further information or data regarding this phenomenon.

SECTION 5. CONCLUSION

Section 5 Conclusion

The aims of this work were to characterise the growth of HL-60 cells within a diffusion chamber *in vivo* and, if possible, to utilise this model as a screening system to assess the ability of various agents to induce the terminal differentiation of HL-60 cells *in vivo*. It was subsequently demonstrated that the growth of HL-60 cells could be propagated in a reproducible manner within a diffusion chamber and that the percentage of spontaneous differentiation could be regulated by the use of a medium which contained serum within the chamber. This work was extended to an *in vivo* study of the differentiating effect of various agents which were known inducers of HL-60 cells *in vitro*. These experiments were performed under conditions in which the pharmacokinetics of each inducer were known. The results demonstrated that agents which were present *in vivo*, at sub-toxic concentrations, could induce the terminal differentiation of the implanted HL-60 cells. This work had extended the work previously reported by other investigators, in that rarely had drug-induced terminal differentiation been induced *in vivo*, and not under conditions of known pharmacokinetics of the various agents; it also suggested that the diffusion chamber could provide a reproducible model with which to study the *in vivo* drug-induced differentiation of HL-60 cells.

This work suggests an alternative strategy for differentiation therapy in patients. Whereas the clinical trial of HMBA (as outlined in section 1.2.3) involved a 120 hour continuous infusion of the drug, it might be possible to administer a higher dose of each agent at short intervals and achieve the induction of terminal differentiation *in vivo*. However, even the most efficient inducer in this system, TMU, only induced just 60% of HL-60 cells to terminally differentiate *in vivo*. Therefore further experimentation with the diffusion chamber system would have to be performed to determine the optimum scheduling regimes so as to maximise the differentiating efficacy of the various agents before they could be considered potential candidates for clinical trial.

The trial which involved HMBA, although it was a good boost for differentiation therapy, might have produced positive results if this agent had been assayed more thoroughly *in vitro*, possibly with the various regimes of washing and dosing as were performed in section 4.6.; and in an *in vivo* system such as the diffusion chamber. This system could have been used to design the scheduling of the drug so as to induce the maximum percentage of differentiation with minimum toxic side effects. The results presented here suggest that TMU would be a potential agent for clinical trial as a differentiating

agent; whereas the other agents tested, the formamides, would not be suitable candidates for trial as differentiating agents since it was not possible to achieve sub-toxic concentrations of these agents *in vivo*.

This work has provided the first reproducible model with which to study, *in vivo*, the drug-induced terminal differentiation of HL-60 cells. The system has been characterised with respect to optimising the conditions required for the growth of the cells and the pahmacokinetics of each agent can also be studied using this technique. Further work could involve the use of this *in vivo* system for the selection of suitable compounds for trial as differentiating agents and also for the determination of regimes of dosing required for each agent.

SECTION 6 APPENDIX

SECTION 6.

APPENDIX 1

6.1. Characterisation of the Differentiation Induced by TPA and RPA.

In order to combine the differentiating effects of phorbol esters and retinoic acid derivatives and to attempt to decrease the tumour-promoting effects of phorbol esters, several retinoic acid esters of phorbol derivatives have been prepared (Sorg et al, 1982). One of these compounds, 12-o-retinoylphorbol-13-acetate (RPA), was found to possess none, or only very weak, tumour-promoting activity (Furstenberg et al, 1982). RPA was subsequently found to induce macrophage differentiation in certain clones of mouse myeloid leukaemia and HL-60 cells (Simantov et al, 1983). Later, Fibach et al (1984) reported that RPA was as potent as TPA with respect to inhibition of cell proliferation and induction of macrophage-specific markers. This compound was therefore also tested in vitro with TPA.

Incubation of HL-60 cells with various concentrations of TPA or RPA induced the terminal differentiation of the

Fig. 6.1. Experiments to determine the relationship between the induction of differentiation and various concentrations of 12-o Tetradecanoylphorbol 13-acetate

Fig. 6.1a Cell numbers measured on days 3 and 5.
Fig. 6.1b Differentiation measured on day 3.
Fig. 6.1c Differentiation measured on day 5.

All figures are the mean values of 4 experiments and the standard error <10%.

Fig 6.1a

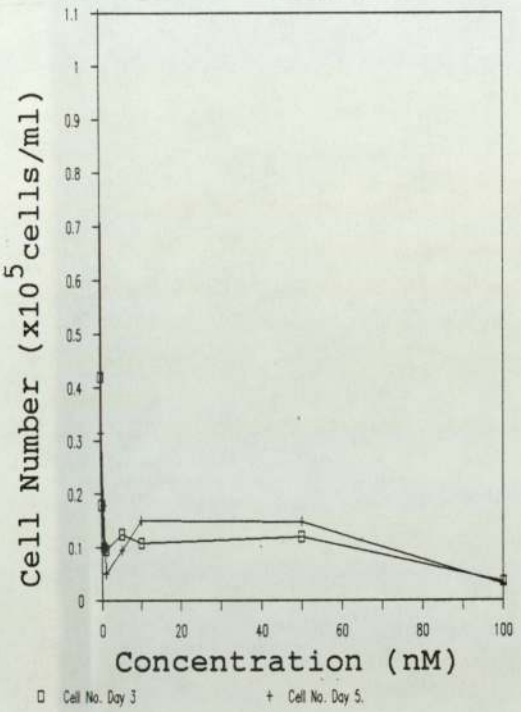


Fig 6.1b

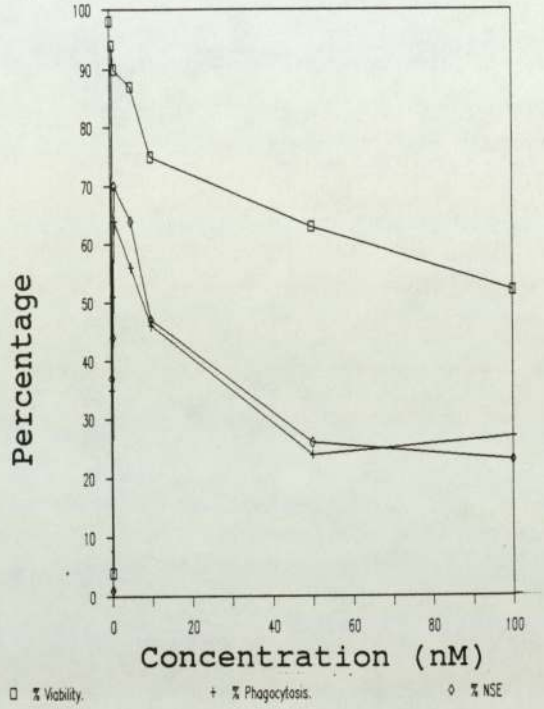


Fig 6.1

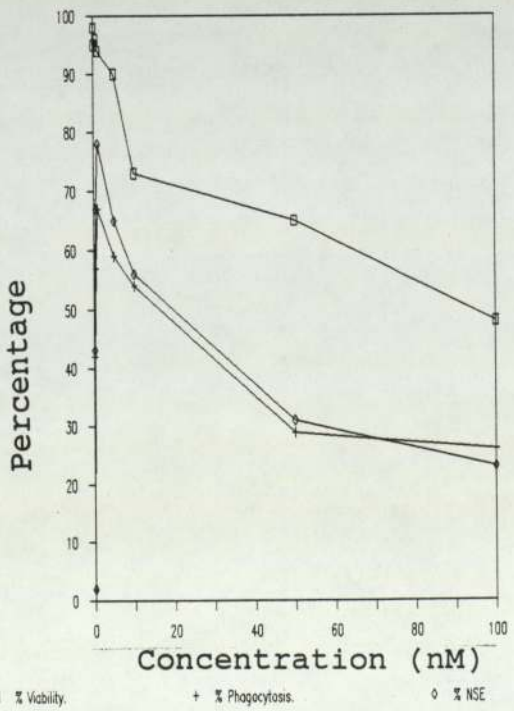


Fig. 6.2. Experiments to determine the relationship between the induction of differentiation and various concentrations of 12-o-Retinoylphorbol 13-acetate.

Fig. 6.2a Cell numbers measured on days 3 and 5.
Fig. 6.2b Differentiation measured on day 3.
Fig. 6.2c Differentiation measured on day 5.

All figures are the mean values of 4 experiments and the standard error was <10%.

Fig 6.2a

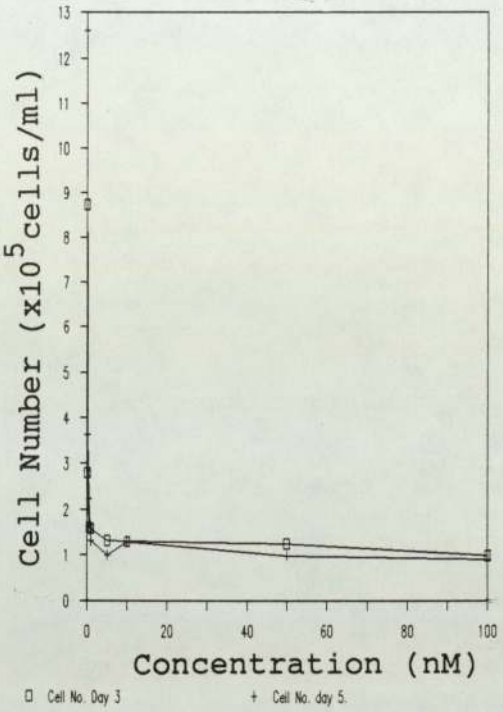


Fig 6.2b

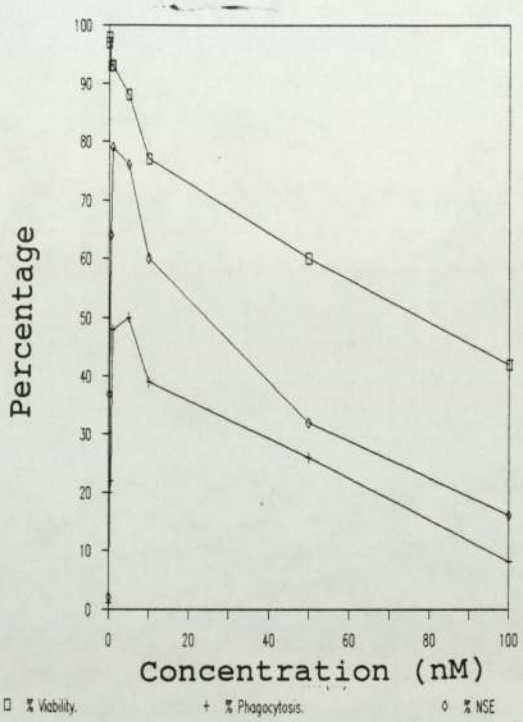
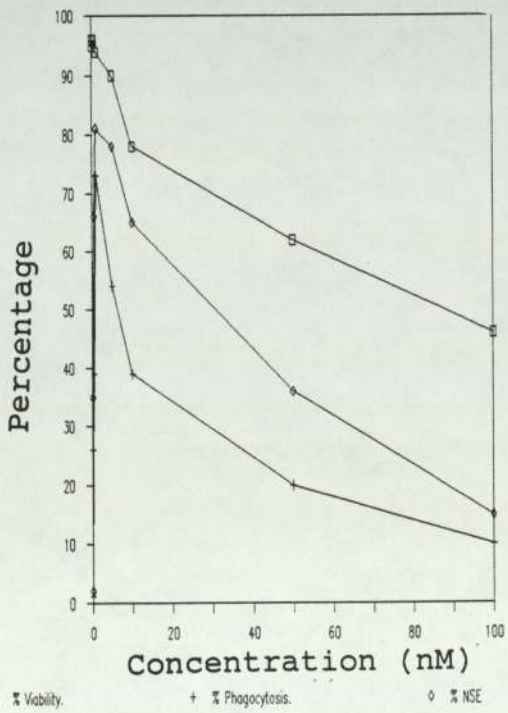


Fig 6.2c



cells to monocyte/macrophage -like cells as previously reported by Fibach et al (1984) (Fig.6.1. and 6.2)

Addition of TPA/RPA to the cells resulted in an almost immediate arrest of cell division. The control cell population underwent 4 cell doublings, whereas the cells treated with TPA or RPA, at concentrations which ranged from 1-100nM, hardly increased in number during the duration of the experiment. Huberman et al (1982) reported that TPA and teleocidin inhibited the growth of HL-60 cells by blocking them from entering the DNA-synthesis phase (S-phase) of the cell cycle which resulted in an accumulation of cells in G1.

The cell viability of the control cell population remained in excess of 90%, but there was a decreased cell viability concomitant with increasing concentrations of TPA or RPA, and at a concentration of 100nM TPA/RPA there were only 50% of remaining cells viable.

Examination of the temporal relationship between the addition of the inducers and the expression of markers of differentiation is shown in figs 6.3 and 6.4. There was an increase in NSE activity to 18% after 24 hours treatment with either of the inducers which reached a maximum of 75% by day 4. The percentage of cells capable of phagocytosing complement-coated yeast particles increased from 1% to 63% after 2 days treatment with the

Fig.6.3. Assay of the effect of 1nM TPA on the differentiation of HL-60 cells at various time periods.

Fig. 6.3a Cell numbers - control and TPA-treated cells.
Fig. 6.3b Percentage of differentiation of the TPA-treated cells.
Percentage of differentiation of the control cells <8% and the cell viability was >90%. Data not shown.

All figures are the mean values of 4 experiments and the standard error was <10%.

Fig 6.3a

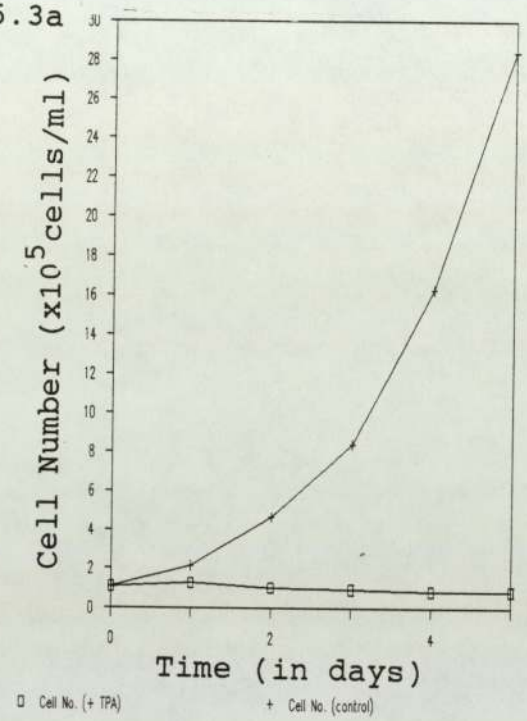


Fig 6.3b

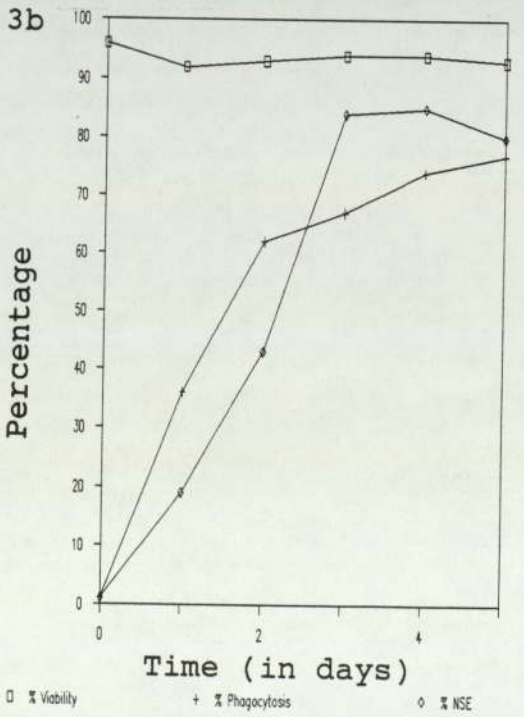


Fig. 6.4. Assay of the effect of 1nM RPA on the differentiation of HL-60 cells at various time periods.

Fig. 6.4a Cell numbers -control and RPA-treated cells.
Fig. 6.4b Percentage of differentiation of the RPA-treated cells
Percentage of differentiation of the control cells <8% and the cell viability was >90%. Data not shown.

All figures are the mean values of 4 experiments and the standard error was <10%.

Fig 6.4a

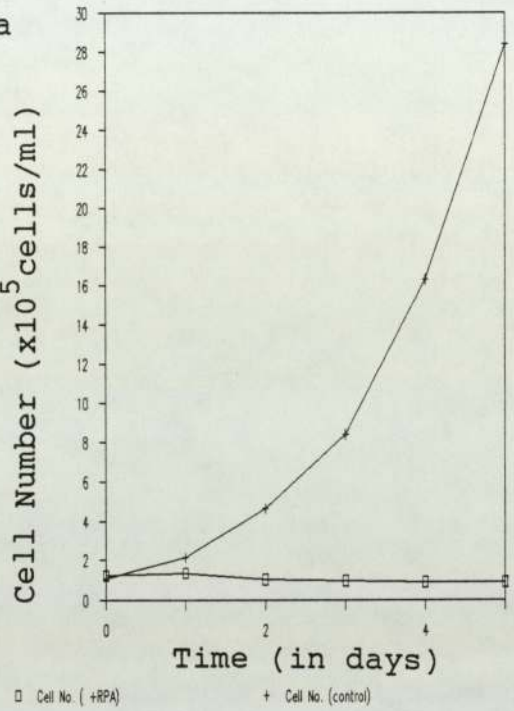
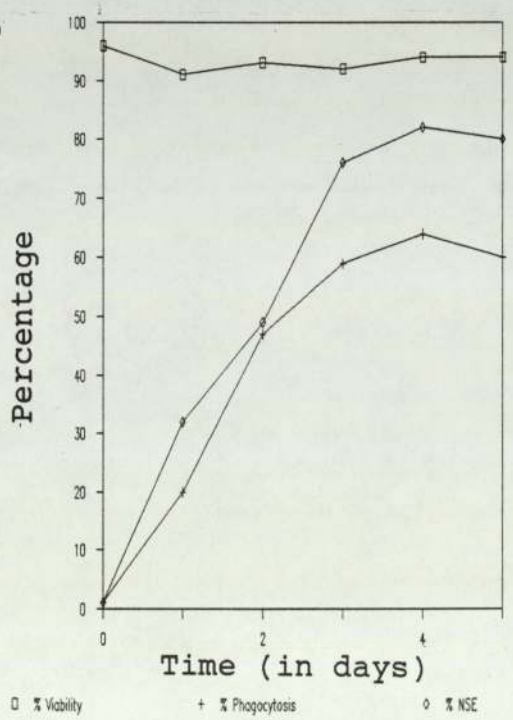


Fig 6.4b



inducers, and this activity reached a maximum by day 6/7. The expression of the C3b complement receptor appears relatively late in the maturation process of myeloid cells (Werb, 1982) and hence the figures obtained for phagocytic activity were always lower than those obtained for NSE activity; when measured on the same day.

The NBT reduction assay was not utilised to assess TPA/RPA-induced HL-60 cell differentiation primarily because this activity was common to both granulocytes and macrophages, but also because Newburger et al (1981) reported that TPA-induced HL-60 cells were not capable of reducing NBT or that there was a very much decreased reaction. Assessment of phagocytic activity was also common to both pathways of HL-60 cell maturation but this was used to conform the presence of mature differentiated cells and was combined with the measurement of NSE activity which was specific for monocyte-macrophage cells.

The optimum concentration of TPA and RPA that was required for the in vitro monocytic differentiation of HL-60 cells was 1nM. This concentration induced >70% of cells to differentiate whilst retaining a cell viability of >85%. This was in concordance with the optimum concentration reported by Huberman et al (1979).

6.2. Determination of the commitment time required by HL-60 cells to differentiate when treated with TPA and RPA.

Reports of the amount of time that TPA has to be present with the cells before they become committed to monocytic differentiation has varied from 20 minutes (Mitchell et al, 1985) to 48 hours (Fibach et al, 1982).

Experiments were therefore carried out to determine how long the cells needed to be incubated with 1nM TPA or RPA to commit them to differentiate, even in the subsequent absence of the inducer (Figs. 6.5. and 6.7.). Differentiation was assessed 72 hours from the start of the experiment with the cells being washed 6 times to remove the inducers before being resuspended in fresh medium. Untreated HL-60 cells were then resuspended in the supernatents obtained from each washing procedure as a "bioassay" to determine whether the TPA/RPA was being successfully removed from the cells by the washing procedure (Fig. 6.6.). To further verify that the TPA/RPA was being removed from the cells, these inducers were added for 5 minutes and then washed out. This was considered too short an exposure time to commit the cells to differentiate and would confirm that the washing procedure was removing the inducers. These cells were

so assessed for differentiation 72 hours after the start of the experiment.

The cells showed a significant increase in monocytic differentiation, as measured by assays of phagocytosis and NSE activity, after only 4-7 hours incubation with the inducers which reached a maximum after 12 hours exposure to TPA/RPA. The cell viability remained in excess of 85% and there was a decrease in cell numbers after 3 hours exposure to the inducers. The cells treated with TPA/RPA for only 5 minutes showed no significant monocytic differentiation suggesting that the washing procedure was sufficient. Previous attempts at this experiment had only washed the cells 3 times before resuspending them in fresh medium; the cells at every timepoint had subsequently shown the same percentage of monocytic differentiation suggesting that the washing procedure had not been successful in removing the inducers from the cells.

Incubation of untreated HL-60 cells in the supernatents obtained from each wash suggested that the majority of the TPA/RPA was removed after 4 washes (Fig. 6.6). The cells showed decreasing amounts of NSE activity with each wash, with the supernatent obtained from the 5th wash inducing only 5% of cells to differentiate.

These findings are in contrast to treatment of HL-60 cells with inducers of granulocytic differentiation which

Fig.6.5.Results of the assay to determine the length of time required to commit HL-60 cells to differentiate using 1nM TPA.

Fig. 6.5a Cell numbers measured 72 hours from the start of the experiment.
Fig. 6.5b Assays of Differentiation.

All figures are the mean values of 4 experiments and the standard error was <10%.

Fig 6.5a

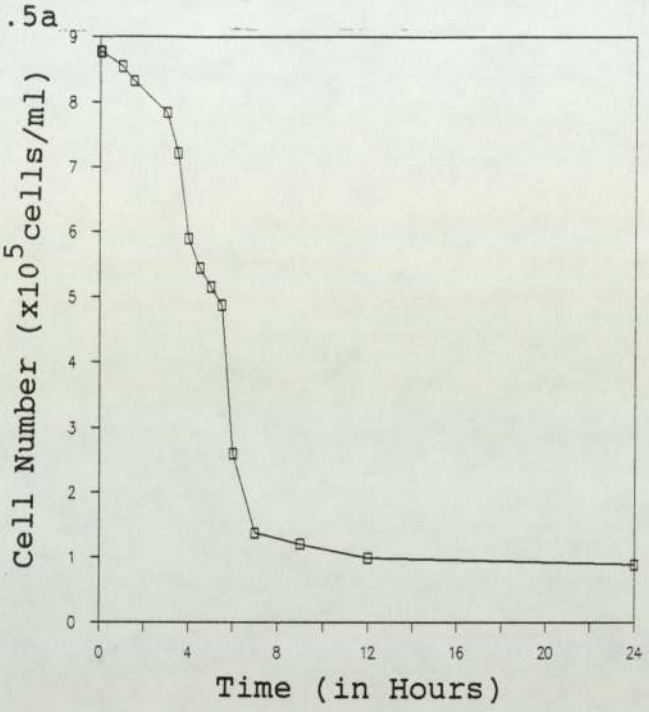


Fig 6.5b

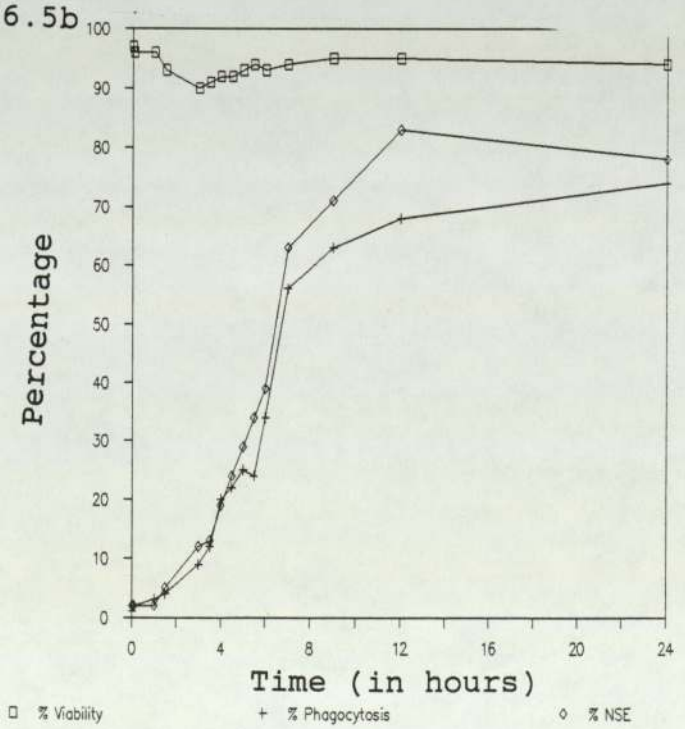


Fig. 6.6. Bioassay of the supernatents obtained from the washing procedure to determine the length of time required to commit HL-60 cells to differentiate using 1nM TPA.

Assays of differentiation measured 72 hours from the start of the experiment.
All figures are the mean values of 4 experiments and the standard error was <10%.

Fig 6.6

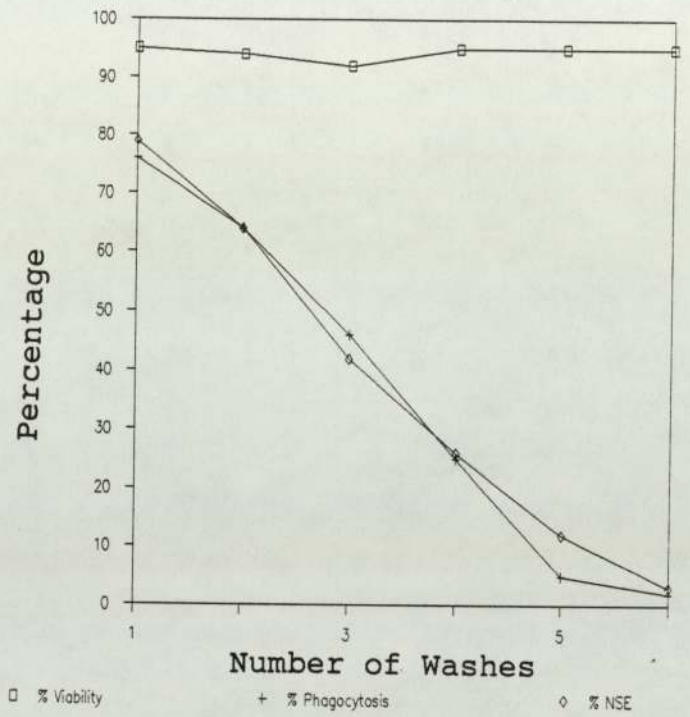


Fig. 6.7. Results of the assay to determine the length of time required to commit HL-60 cells to differentiate using 1nM RPA.

Fig. 6.7a Cell numbers measured 72 hours from the start of the experiment.
Fig. 6.7b Assays of differentiation.

All figures are the mean values of 4 experiments and the standard error was <10%.

Fig 6.7a

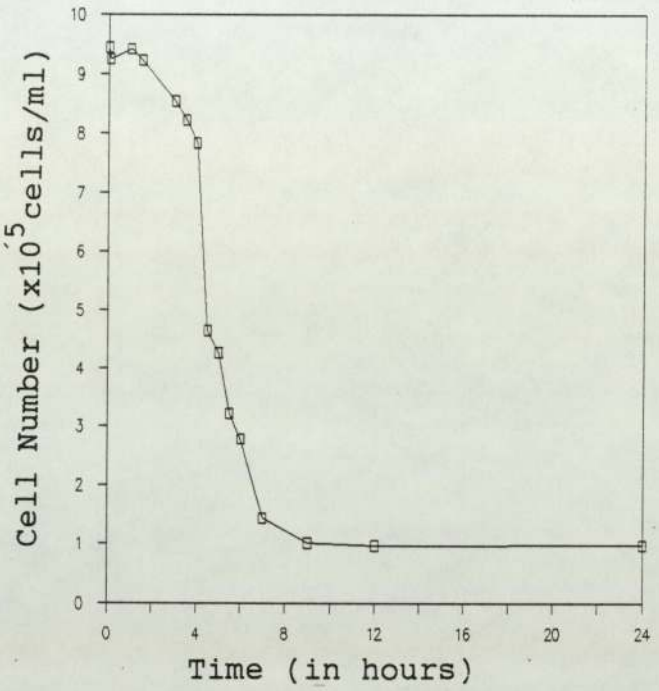
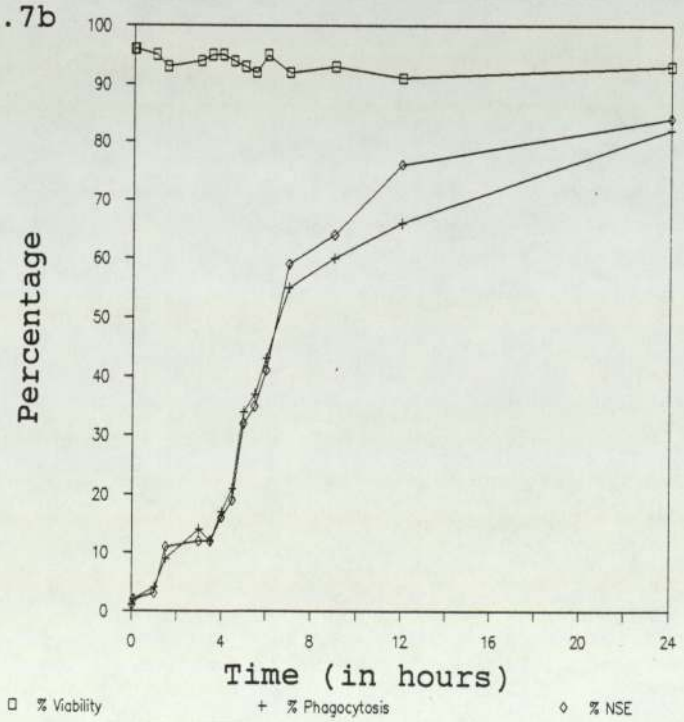


Fig 6.7b



had to be present for 24 hours before a significant percentage of cells were induced to differentiate (Breitman et al, 1980) (see section 4.2). HL-60 cells, when treated with the inducers of monocytic differentiation, such as TPA/RPA, become committed to differentiate very rapidly without the need for cell replication (Territo et al, 1981). The commitment time required by this subline of HL-60 cells was 4-7 hours.

6.3. The Effects of Pretreating HL-60 Cells with Retinoic Acid on the Commitment Time

Attempts were made to decrease the commitment time required for monocytic differentiation because as short a commitment time as was attainable was considered important for later in vivo work. Fibach et al (1982) had demonstrated that a short exposure of 6-24 hours to retinoic acid or other inducers of granulocytic differentiation. including DMSO, butyric acid and HMBA, caused an enhancement of the response of HL-60 cells to the macrophage-inducing effects of phorbol esters. In addition, lower concentrations of phorbol esters were required and the various macrophage-specific markers appeared sooner in cells that had been pretreated with

granulocytic inducers, provided that exposure to the inducer was shorter than the commitment time required for that particular inducer. This data suggested that the initial portion of commitment and differentiation to either the granulocytic or monocytic pathways were similar.

Pretreatment of HL-60 cells with 10nM retinoic acid for 18 hours with the subsequent addition of TPA/RPA resulted in a decrease in the commitment time to <30 minutes (Figs 6.8 and 6.9). There was a small but significant increase in the percentage of NSE activity and phagocytic capability after only 30 minutes exposure to the inducers.

The simultaneous addition of TPA and retinoic acid induced a higher percentage of phagocytic activity than exposure to either of the inducers separately (Fig 5.1.) suggesting there may have been some synergism between TPA and retinoic acid - possibly the induction of both granulocytic and monocytic differentiation which are both capable of immune phagocytosis. This was further supported by the percentage of NSE activity which was >60% for the TPA-treated cells but was only approximately 42% for the cells treated with both TPA and retinoic acid - suggesting that the majority of the cells were monocyte-macrophage cells but there were some retinoic acid -induced granulocytes present as indicated by the increased phagocytic activity of approximately 55%.

Fig.6.8..Results of the assay to determine the length of time required to commit HL-60 cells to differentiate using 1nM TPA after pretreatment of the cells with 10nM retinoic acid.

Fig. 6.8a Cell numbers measured 72 hours from the start of the experiment.
Fig. 6.8b Assays of differentiation.

All figures are the mean values of 4 experiments and the standard error was <10%.

Fig 6.8a

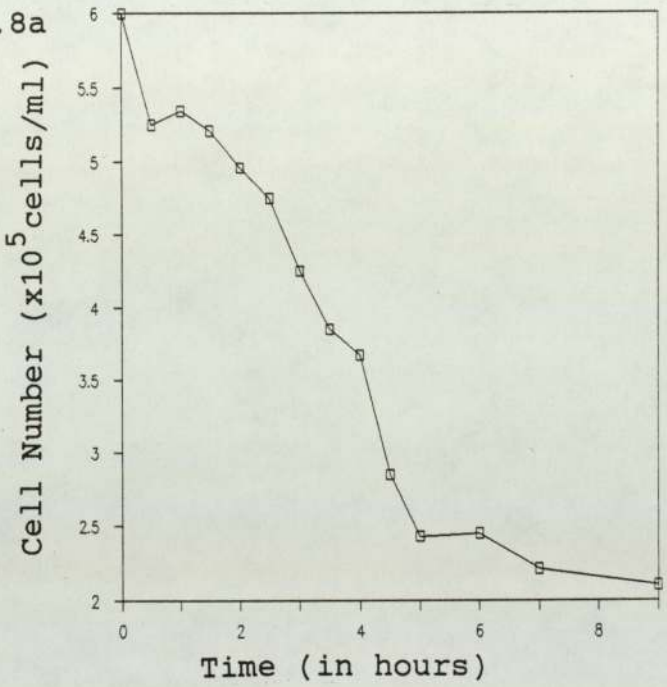


Fig 6.8b

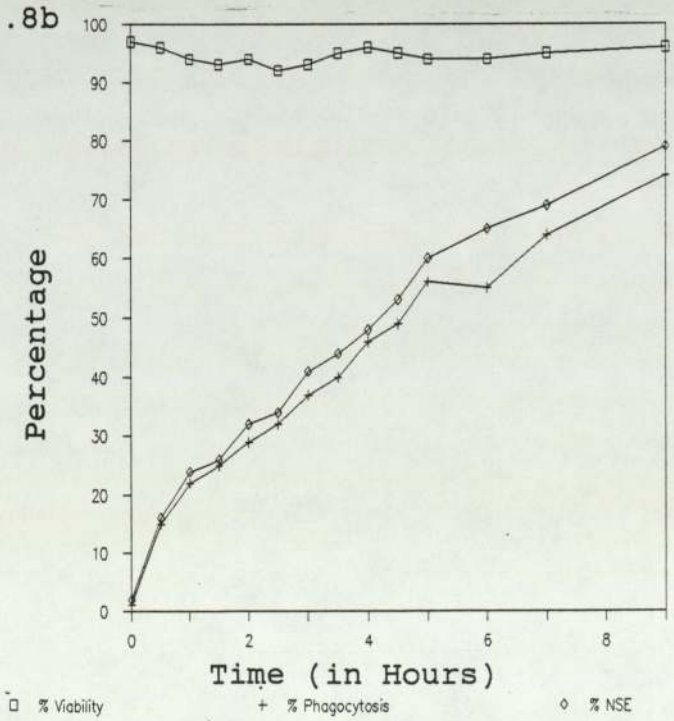


Fig.6.9. Results of the assay to determine the length of time required to commit HL-60 cells to differentiate using 1nM RPA after pretreatment of the cells with 10nM retinoic acid.

Fig. 6.9a Cell numbers measured 72 hours from the start of the experiment.
Fig. 6.9b Assays of differentiation.

All figures are the mean values of 4 experiments and the standard error was <10%.

Fig 6.9a

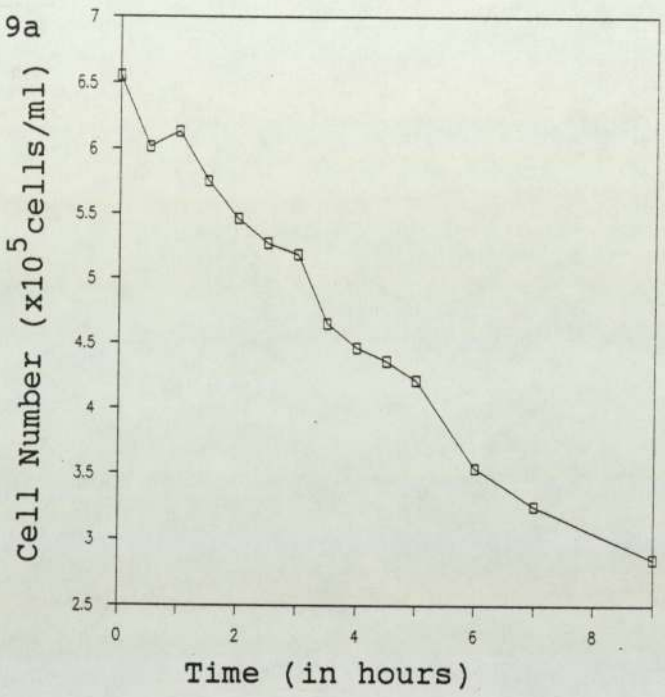
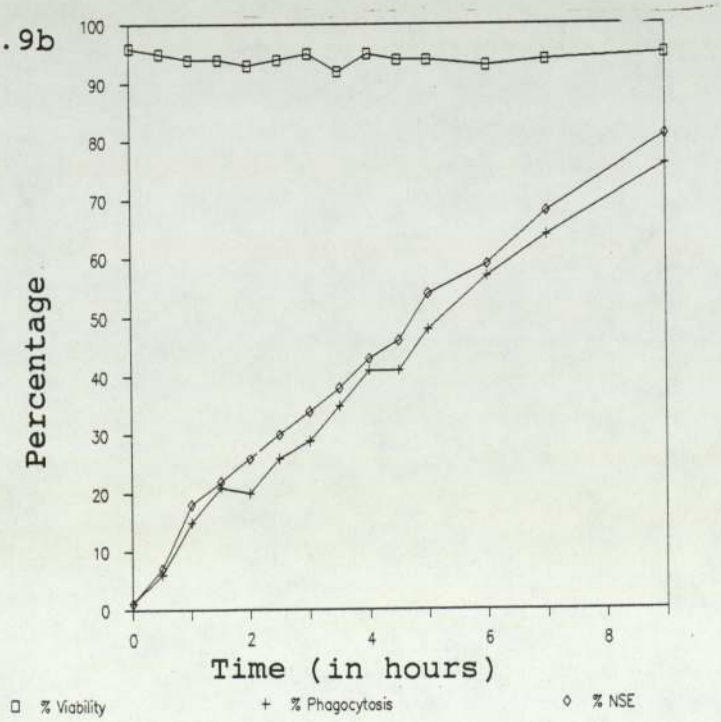


Fig 6.9b



The viability of the cells treated with either TPA or retinoic acid alone was in excess of 95%, whereas those cells that were treated with both TPA and retinoic acid simultaneously had a decreased viability of 85%.

6.4. Administration of TPA In Vivo.

To determine a suitable dose for administration to the mice, 0.1mg TPA was initially dissolved in 1ml DMSO and this was further diluted using arachis oil to the required concentration. This was injected either ip. or sc. into the leg muscle into BDF1 mice at concentrations which ranged from 0.05-20ug/mouse. There were no deaths and only the mice receiving 10 and 20µg TPA showed any toxic effects. It was not possible to determine the pharmacokinetics of TPA during the time of this thesis work.

Table 5.1 The simultaneous addition of TPA and RA to HL-60 cells.

Inducer	No.c/ml. (x100)	% Viability	% Phagocytosis	% NSE
Control	5800	97.3	1	2
TPA alone	1230	95,1	48	62
RA alone	4350	96.0	46	2
TPA and RA	1250	85.7	56	42
RA then TPA	2450	85.2	65	65

The HL-60 cells were treated with either 1nM TPA, 10nM RA, 1nM TPA and 10nM RA together or 10nM RA for 18 hours then 1nM TPA. The percentage of differentiation was assessed 72 hours from the start of the experiment. All figures are the mean values of 4 experiments.

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